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Enzyme Inhibition in Relation to Chemotherapy.\* (17313)

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Studies on enzyme inhibitors are frequently reported in terms of the percent inhibition in comparison with a control containing no inhibitor. It is the purpose of the present paper to show that this approach is frequently in danger of giving misleading results for the reason that with some inhibitors the percent inhibition is a function of the enzyme concentration. As will be pointed out in the discussion, the existence of such inhibitors is of considerable importance from the standpoint of pharmacology, chemotherapy and related fields, and it is therefore of some importance to have an effective experimental method for their recognition.

The phenomenon which we propose to describe is essentially the outcome of an "irreversible" reaction between enzyme and inhibitor, so that for all practical purposes the enzyme is effectively titrated or stoichiometrically combined with a definite amount of inhibitor. From the theoretical standpoint the "irreversible" reaction between enzyme and inhibitor may occur in a variety of ways; the simplest case is one in which the enzyme-inhibitor complex is theoretically reversible

but has a dissociation constant so small that the combination seems irreversible. We shall therefore refer to this type of inhibition as *pseudo-irreversible*. Equally possible is a situation in which the enzyme reacts with an inhibitor in a truly irreversible manner, that is to say, the enzyme is converted to a form which cannot be converted back into active enzyme. In either case, the amount of enzyme inactivated will depend not only upon the amount of inhibitor but upon the amount of enzyme present. Regardless of the exact nature of the irreversibility, it can be recognized very simply by means of an experimental test: by determining the rate of reaction at different enzyme concentrations plus or minus inhibitor, it should be found that in the case of the controls, the rate is proportional to the enzyme concentration, so that a straight line through the origin is obtained when rate is plotted against enzyme amount. In the presence of a reversible inhibitor a straight line through the origin also results, but the slope of the line is less than in the case of the control. In the case of an irreversible inhibitor, the slope of the line is the same as that of the control but it will pass through the X-axis to the right of the origin by an amount that is proportional to the amount of inhibitor (see Bain<sup>1</sup>). This graphic test is not de-

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A preliminary report of this work was given at the Annual Meeting of the American Association for Cancer Research, April 16 and 17, 1949, (*Cancer Research*, 1949, 9, 602).

<sup>1</sup> Bain, J. A., *PROC. SOC. EXP. BIOL. AND MED.*, 1949, 72, 9.

pendent upon any theoretical assumptions as to the nature of the irreversibility and is of considerable value in the interpretation of inhibitor data whether obtained with pure enzymes, homogenates, minces, slices or whole cells. It is of interest that the curve for the pseudo-irreversible type of inhibitor is simply one of a family of curves, and that the curve for the reversible type can be obtained from the same general equation that is derived from the familiar Michaelis-Menten equation in the theoretical part of this paper. With the same equation it can be shown that when the dissociation constant of the enzyme-inhibitor complex has an intermediate value, the plot of activity against enzyme amount yields a line that is intermediate between the two types described above. Since many of the recently discovered enzyme inhibitors appear to fall in this category the test may be of value in understanding their action, since in these cases the per cent inhibition also depends upon the enzyme concentration.

The fact that inhibitors may fall into 3 broad categories depending upon the relationship between the dissociation constant of the enzyme-inhibitor complex and the concentration of enzyme has been emphasized previously by Straus and Goldstein<sup>2</sup> and by Goldstein.<sup>3</sup> They pointed out that previous mathematical treatments based on the Michaelis-Menten equation assumed "that the concentration of enzyme centers is constant and so small compared with the concentration of any substance with which it may combine that it may be neglected." They called attention to the fallacy in this assumption and developed the Michaelis-Menten equation along lines that include the factor of enzyme concentration.<sup>†</sup>

Our own observations and those of Bain<sup>1</sup> are in accord with the conclusions of Straus and Goldstein<sup>2,3</sup> and are believed to supplement their work by presenting a simple graphic method for recognizing the situations in which the older mathematical treatments are more or less inadequate. The graphic

method is accompanied by a mathematical analysis in which one of the components of the graph (rate) is obtained as a function of the other component (enzyme amount) in an equation involving other variables (substrate amount, inhibitor amount, enzyme-substrate dissociation constant, and enzyme-inhibitor dissociation constant). The graphic method involves no assumptions as to the mechanism of inhibitor action, but in conjunction with the equation given it permits one to obtain an apparent dissociation constant for the enzyme-inhibitor complex. The word "apparent" is used here because in the case of an inhibitor which gives a line that intercepts the X-axis it would not be possible to decide from the graph whether the inhibitor was pseudo-irreversible (*i.e.*, dissociation constant of enzyme-inhibitor complex very small) or truly irreversible. In addition, the apparent dissociation constant will be affected by the specificity of the inhibitor and the presence of other compounds with which it may combine. It must be emphasized that the main purpose of the graph is not to determine the dissociation constants but to distinguish between the reversible type of inhibitor and the "irreversible" inhibitors. It is believed that the graph will permit this distinction regardless of the presence of interfering compounds and no evidence to the contrary has been obtained.

The data given are for illustrative purposes only, and have no intrinsic interest except insofar as they illustrate the principles described above. For this work, succinoxidase was employed as the test system because previous experience<sup>4</sup> showed that it could be

<sup>2</sup> Straus, O. H., and Goldstein, A., *J. Gen. Physiol.*, 1943, **26**, 559.

<sup>3</sup> Goldstein, A., *J. Gen. Physiol.*, 1944, **27**, 529.

<sup>†</sup> The important contributions by Michaelis and Menten, by Haldane, and by Lineweaver and Burk (referred to by Straus and Goldstein<sup>2,3</sup>) provide the foundation for the further expansion of the basic equations to include the amount of enzyme as a variable. The older equations are still very useful as well as appropriate in situations in which competitive inhibition between reversible inhibitors and substrates occurs since in these situations variation in enzyme amount does not affect the per cent inhibition. The newer treatment is more appropriate in the case of the pseudo-irreversible inhibitors.

inhibited by two types of inhibitors, the malonate-type and the type that combines with sulfhydryl groups. Whole homogenates were used as in the previous work, in which it was shown that the sulfhydryl inhibitors acted upon an essential group that could be protected by malonate, although malonate would not protect other enzymes against the sulfhydryl inhibitors. It thus seems clear that the sulfhydryl reagents used in this study inactivate succinoxidase by direct interaction with the enzyme.

**Experimental. Test System.** In these studies the succinoxidase system was employed. In all experiments a 10% water homogenate of liver was prepared in the usual manner and used as a source of the succinic dehydrogenase after diluting to 2.5%. The basal reaction mixture used was the same in all cases and prepared as previously described.<sup>5</sup> The concentration of inhibitors and homogenate are in the corresponding plots of the experimental data. The total volume of material in each flask was 3 ml and the temperature of the reaction 38°C. The rate of oxygen uptake per 10 minutes was determined on the basis of 4 successive 10-minute periods. With all inhibitors except oxalacetate in experiments reported in Fig. 1, the homogenate was incubated for 30 minutes at room temperature with the inhibitor before the addition of succinate, since some inhibitors do not react instantly with the enzyme, and are affected by the presence of the substrate.

**Compounds Tested.** The itaconic acid was obtained from the Chas. Pfizer Company while the malonic acid and quinone were obtained from the Eastman Kodak Company. Oxalacetic acid was prepared from sodium ethyl oxalacetate.

**Results. Effect of Enzyme Concentration.** The reaction velocity for the enzymatic dehydrogenation of succinate to fumarate could be measured in terms of the oxygen taken up in successive 10-minute intervals, since the

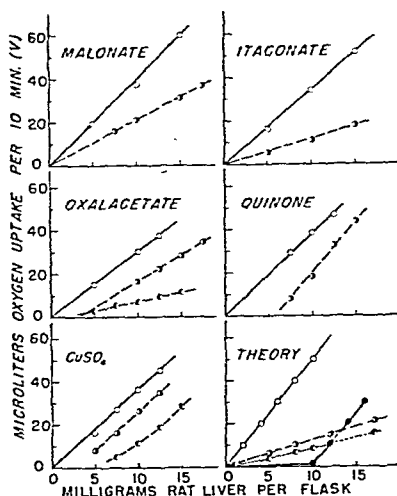


FIG. 1.

The reaction velocity as measured by oxygen uptake for the enzymatic dehydrogenation of succinate to fumarate in the presence and absence of inhibitors. The concentration of malonate was  $1 \times 10^{-3}M$ ; oxalacetate was  $6.7 \times 10^{-5}M$  and  $3.4 \times 10^{-5}M$ ; copper sulfate was  $1.3 \times 10^{-4}M$  and  $2.6 \times 10^{-4}M$ ; itaconate was  $5 \times 10^{-2}M$ ; quinone was  $3.3 \times 10^{-4}M$ . In all cases the concentration of succinate was  $5 \times 10^{-2}M$ . In the curve systems labeled malonate, oxalacetate,  $CuSO_4$ , itaconate and quinone, the broken lines represent activity in the presence of the inhibitors. The system of curves labeled theory were calculated from equation (13) assuming for all cases  $S = 5 \times 10^{-2}M$  and  $K_s = 10^{-3}$ : for the solid curve  $I_t = 0$ ; for the broken curve  $K_i = 10^{-4}$ ,  $I_t = 1 \times 10^{-3}M$ ; for the dashed curve  $K_i = 10^{-6}$ ,  $I_t = 5 \times 10^{-5}M$ ; for the double lined curve  $K_i = 10^{-9}$ ,  $I_t = 1 \times 10^{-5}M$ .

succinic dehydrogenase is the limiting factor in the succinoxidase system when both cytochrome c and cytochrome oxidase are present in excess. It was established that the reaction rate in the absence of inhibitors was proportional to the amount of homogenate. Since a different liver homogenate was used for each experiment, it was necessary to include controls each time an inhibitor was tested. It may be seen in Fig. 1 that the control data in each instance (open circles) yielded a satisfactorily straight line that passes through the origin.

However, when such data were obtained with various inhibitors present and were plotted in the same manner, significant differences between the inhibitors were revealed insofar as they were affected by enzyme concentration.

<sup>4</sup> Potter, V. R., and Du Bois, K. P., *J. Gen. Physiol.*, 1943, 26, 391.

<sup>5</sup> Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, 1943, 149, 217.

In the presence of malonate or itaconate (Fig. 1), which are structurally similar to succinate, the rate of dehydrogenation was also proportional to the enzyme concentration, but the slope of the line was less than in the case of the controls. The per cent inhibition was thus the same at any concentration of enzyme and depended solely upon the concentration of inhibitor since the succinate concentration was constant.

In contrast to the data obtained with malonate and itaconate, oxalacetate, which also bears a close structural relationship to succinate, yielded data that do not fall on a line through the origin, but rather on a line which appears to intercept the enzyme axis (Fig. 1).

Even more striking are the results with quinone and copper (Fig. 1). Here the data fell on lines that tend to be straight and parallel to the control. With these inhibitors the per cent inhibition is clearly related not only to the inhibitor concentration but also to the enzyme concentration.

**Interference Phenomenon with Irreversible Inhibitors.** In earlier studies on succinoxidase<sup>1</sup> it was shown that the inhibition produced by malonate was instantaneous and independent of the order of addition of substrate and inhibitor. This was in contrast to results obtained with agents like quinone and copper, which produced a slowly increasing per cent inhibition that was strongly affected by the presence or absence of succinate or, indeed, of malonate. In the presence of succinate or malonate, the sulfhydryl inhibitors react more slowly with the enzyme than they do when both of these compounds are absent. A more detailed experiment on this point is reported in Fig. 2. In this experiment a series of Warburg flasks was prepared with the usual reaction mixture but no succinate, and with an equal amount of tissue in each flask. At zero time copper in the form of  $\text{CuSO}_4$  was added to all of the flasks, and at successive intervals succinate was added from the side arm and oxygen uptake was measured. The continuous lines represent the rate of oxygen uptake during successive 10-minute time intervals after the

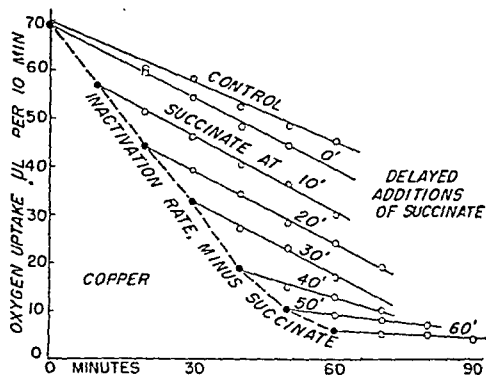


Fig. 2.

Rate of inactivation of succinic dehydrogenase by copper in the presence and absence of succinate. The final concentrations of succinate and of  $\text{CuSO}_4$  were 0.067 M and  $3.3 \times 10^{-5}$  M, respectively. The solid circles represent extrapolated points. The open circles are experimental points.

addition of succinate, and show that when succinate was present no inactivation of the enzyme by the copper occurred, since the decline in rate was no greater than in the controls. The broken line indicates the rate of enzyme inactivation by copper when no succinate was present, and shows that a progressive decrease in active enzyme occurred as long as succinate was absent, but as soon as succinate was added the process of inactivation ceased abruptly. This experiment shows that succinate *interferes* with the reaction between copper and succinic dehydrogenase, while copper once combined with the enzyme cannot be effectively displaced by succinate. When larger amounts of sulfhydryl inhibitors were added to the succinoxidase system, it was possible to progressively inhibit the enzyme even in the presence of succinate, although not as rapidly as in the absence of succinate. It is clear that copper and succinate compete for the enzyme and that their reactions with it are *mutually exclusive* as suggested earlier in the case of quinone.<sup>4</sup> For the present we shall refer to this type of effect as an "interference phenomenon" and will not attempt to describe it as competitive inhibition. But it must be pointed out that the latter term is a purely operational concept that is at present restricted to reversible inhibitors. The experimental approach indi-

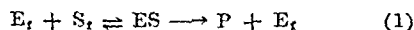
cated in Fig. 2 provides a means of studying competition between substrates and irreversible inhibitors during the course of the reaction between the inhibitor and the enzyme as opposed to competition during the measurement of enzyme activity. It is proposed that this kind of competition be called *interference* in order to avoid needless confusion in terminology.

*Theoretical.* To derive an expression in which ES is expressed as a function of  $E_t$ ,  $S$ ,  $I_t$ ,  $K_s$ , and  $K_i$ , the symbols listed below were used:

- $E_t$  = Total Enzyme Concentration.
- $E_r$  = Uncombined Enzyme Concentration.
- $EI$  = Enzyme-Inhibitor Complex Concentration.
- $ES$  = Enzyme-Substrate Complex Concentration.
- $S$  = Total Substrate Concentration.
- $S_r$  = Uncombined Substrate Concentration.
- $P$  = Product Concentration.
- $K_i$  = Enzyme-Inhibitor Complex Dissociation Constant.
- $K_s$  = Enzyme-Substrate Complex Dissociation Constant.
- $I_t$  = Total Inhibitor Concentration.
- $I_r$  = Uncombined Inhibitor Concentration.
- $k$  = Velocity Constant.

The equation was developed along lines similar to the familiar Michaelis-Menten equation but includes more variables.<sup>†</sup> The following assumptions were made:

(a) The reaction between enzyme and substrate or inhibitor can be formulated as in equations (1) and (2):



(b) Where  $(S)$  is very large,  $(S_r)$  is assumed to equal  $(S)$  and  $(E_r)$  approaches zero.

(c) The total inhibitor concentration is equal to the sum of the uncombined and combined forms.

$$(I_t) = (I_r) + (EI) \quad (3)$$

(d) The total enzyme concentration is the sum of the uncombined enzyme, that combined with the inhibitor and that combined with the substrate

$$(E_t) = (E_r) + (EI) + (ES) \quad (4)$$

An equation in which  $ES$  is expressed as a function of  $E_t$ ,  $S$ ,  $I_t$ ,  $K_s$  and  $K_i$  may now be derived.

From expressions (1) and (2) and the mass law one can write:

$$\frac{(E_r) (S_r)}{(ES)} = K_s \quad (5)$$

$$\frac{(E_r) (I_r)}{(EI)} = K_i \quad (6)$$

By substituting the value of  $(EI)$  from (6) into (3) and rearranging one obtains expression (7).

$$(I_r) = (I_t) / \left[ 1 + \frac{(E_r)}{K_i} \right] \quad (7)$$

If one substitutes  $(I_r)$  of expression (7) into (6), solves the resulting expression for  $(EI)$  and substitutes what is then equivalent to  $(EI)$  into (4) one arrives at expression (8).

$$\frac{(E_t) (I_t)}{K_i} + (ES) = (E_r) + \frac{(E_r)}{1 + \frac{(E_r)}{K_i}} \quad (8)$$

Since from (b),  $(E_r)$  is small, (8) reduces to (9)

$$\frac{(E_r) (I_t)}{K_i} + (ES) = (E_r) \quad (9)$$

If one solves equation (5) for  $(E_r)$  and substitutes the result into (9) one arrives at (10).

$$(E_t) = \left[ \frac{(I_t)}{1 + \frac{K_s (ES)}{K_i (S)}} \right] \left[ \frac{K_s (ES)}{K_i (S)} \right] + (ES) \quad (10)$$

By multiplying through equation (10) by the term  $1 + [K_s (ES)]/[K_i (S)]$  and rearranging and solving for  $(ES)$  by the quadratic formula one obtains expression (11), in which  $(ES)$  is expressed as a function of  $(E_t)$ .

$$(ES) = - \left[ \frac{K_i (S)}{2K_s} + \frac{(I_t)}{2} - \frac{(E_t)}{2} \right] \pm \sqrt{\left[ \frac{K_i (S)}{2K_s} + \frac{(I_t)}{2} - \frac{(E_t)}{2} \right]^2 + \frac{K_i (S) (E_t)}{K_s}} \quad (11)$$

Since the velocity of reaction ( $V$ ) is equal to the product of the concentration of the enzyme-substrate complex and the velocity constant for its conversion to the product, then:

$$V = k (ES) \quad (12)$$

or if one multiplies through expression (11) by  $k$  and substitutes  $V = k (ES)$  one obtains†

$$V = -k \left[ \frac{K_1 (S)}{2(K_s)} + \frac{(I_t)}{2} - \frac{(E_t)}{2} \right] \\ \pm k \sqrt{\left[ \frac{K_1 (S)}{2K_s} + \frac{(I_t)}{2} - \frac{(E_t)}{2} \right]^2 + \frac{K_1 (S) (E_t)}{K_s}} \quad (13)$$

The velocity is thus expressed in terms which can be experimentally determined.

We may now consider various tests of the equation. If one lets  $(I_t)$  equal 0, then equation (13) reduces to the following form:

$$V = \frac{-k(S) K_1}{2K_s} + \frac{k(E_t)}{2} + \frac{k(S) K_1}{2K_s} + \frac{k(E_t)}{2} \\ V = k(E_t) \quad (14)$$

For large values of  $(S)$  and no inhibitor, it follows from expression (4) that  $(E_t)$  equals  $(E_s)$ ; hence expression (14) is in agreement with (12). Expression (14) is the equation of a straight line of slope  $k$  passing through the origin. It is also what is experimentally observed when values of  $V$  are plotted against corresponding values of  $(E_t)$  in the absence of inhibitor (Fig. 1). The slope is the

† On the basis of the same assumption made in deriving equation (11), Goldstein<sup>3</sup> has developed the following expression:

$$I' = S' \left[ \frac{1-a}{a} \right] + (1-a) E_t'$$

wherein  $I'$ ,  $S'$  and  $E_t'$  represent the "specific concentrations" of  $I$ ,  $S$  and  $E_t$  and  $a$  is the fractional activity of the enzyme. If one multiplies through the expression of Goldstein by the dissociation constant,  $K_1$ , of the enzyme-inhibitor complex, converts the specific concentrations to absolute concentrations and substitutes  $ES/E_t$  for  $a$ , then on solving the resulting equation for  $(ES)$  in terms of  $I_t$ ,  $K_1$ ,  $K_s$  and  $E_t$  one also arrives at expression (11) which was used in developing expression (13).

velocity constant for conversion of the enzyme-substrate complex to the product.

For small values of  $K_1$ , i.e. where the binding of inhibitor with enzyme is great compared to that of the substrate with enzyme, the terms of equation (13) containing  $K_1$  approach zero and the equation approaches the form of a straight line with a slope  $k$  and an intercept on the enzyme axis. Such an inhibitor virtually "titrates" the enzyme.

$$V = \frac{-k}{2} \left[ (I_t) - (E_t) \right] \pm k \sqrt{\left[ \frac{(I_t) - (E_t)}{2} \right]^2} \\ V = k(E_t) - k(I_t) \quad (15)$$

However equation (14) only approaches the form of (15) since the term containing  $K_1$  only approaches zero. When equation (14) is plotted using various values of  $K_1$ , a system of curves is obtained as shown in Fig. 1, theoretical curves. For values of  $K_1$  that approach the magnitude of  $K_s$ , i.e.  $10^{-4}$  compared to  $10^{-2}$ , a straight line through the origin is obtained. For smaller  $K_1$  values a curved line is obtained and for very small values, i.e.,  $10^{-9}$ , a curve is obtained which rapidly approaches a straight line at a point equal to  $kI_t$  and has a slope equal to  $k$ .

These plots may assist in the interpretation of the experimental data. Malonate with a  $K_1$  equal to  $10^{-4}$  gives a straight line through the origin. Itaconate, a less effective inhibitor, is of the same nature. Copper and quinone which combine with sulfhydryl groups probably have very small  $K_1$  values, while oxalacetate,<sup>6</sup> an intermediate case ( $K_1$  near  $10^{-6}$ ), occupies an intermediate position.

Thus it is possible to explain the nature of the experimental curves relating velocity of reaction to enzyme concentration on the basis of the degree of binding of the enzyme-inhibitor complex relative to the enzyme-substrate complex. This approach also provides an experimental method for determining when one is justified in using the Michaelis-Menten equation to calculate values of  $K_1$  or when a more exact expression is necessary.

<sup>6</sup> Pardee, A. B., and Potter, V. R., *J. Biol. Chem.*, 1948, **176**, 1085.

If a plot of data representing the change of velocity of reaction with changes in enzyme concentration appears to intercept the enzyme axis when extrapolated to zero velocity, then the amount of inhibition will vary with enzyme concentration. In such a case the value of  $K_i$  determined from the Michaelis-Menten equation will not be a constant but will vary depending on the enzyme concentration used in the experiment and a more exact expression than the Michaelis-Menten equation is necessary for calculating  $K_i$ . We have not attempted to derive such an expression because in the case of "titration type" curves the results can be due either to high affinity or true irreversibility as explained earlier.

The analysis represented by equation (11) is incomplete insofar as it does not encompass the effect of the presence of substances other than the enzyme that can combine with the inhibitor. It is clear that the more specific is the inhibitor the less important these considerations will be.

**Discussion.** The main purpose of this paper is (1) to show that in the case of "irreversible" inhibitors, regardless of the nature of the irreversibility, the degree of enzyme inhibition depends upon the enzyme concentration and (2) to consider the implications of this fact. The first question that arises is whether the phenomenon of decreasing per cent inhibition with increasing enzyme amount is a general phenomenon for high affinity inhibitors, or whether the data presented here are merely due to the fact that the high affinity inhibitors are non-specific and are used in a whole homogenate. We believe that the phenomenon is a general one and that increasing numbers of examples will be found (*cf.*

Bain<sup>1</sup>).<sup>†</sup> It is difficult to see how the presence of the other SH groups in the homogenate could produce the type of curve seen with copper or quinone in contrast to the malonate curve, since the ratio between succinoxidase SH groups and "other" SH groups would remain constant as the amount of homogenate was increased. The enzyme system and inhibitors chosen for this study are probably not the best possible examples for illustrative purposes, but the data and conclusions are strongly supported by the accompanying paper on cholinesterase by Bain.<sup>1</sup> In the case of any "irreversible" inhibitor the degree of inhibition produced at any given molarity will depend upon the concentration of enzyme and upon the concentration of other substances that will combine with the inhibitor. This fact applies to any inhibitor, since it can never be assumed that an inhibitor, even though it is known to be highly specific, does not react with unknown constituents in living cells or in preparations therefrom. The reaction with unknown constituents becomes of great importance if it is irreversible. However, the occurrence of side reactions does not invalidate the conclusion that copper and quinone in effect irreversibly inactivate succinoxidase. This inactivation cannot be reversed by succinate, (which has a lower affinity for the enzyme than copper does) but can be reversed by glutathione,<sup>7</sup> which has an affinity for copper similar to that of the enzyme (*cf.* also Barron and Kalnitsky.<sup>8</sup>) This inhibition is therefore considered to be pseudo-irreversible, *i.e.* it has a dissociation constant, but the constant is so small that the percent inhibition varies with the amount of enzyme.

Oxalacetate, which is an intermediate case, is a potent inhibitor for succinoxidase and is not known to inhibit any other enzyme (its action in the malic dehydrogenase system is an equilibrium effect.)

It is thus concluded that the results are not artifacts and that their significance may be examined.

From the standpoint of studies in enzyme

<sup>†</sup> Note added Oct. 24, 1949: Since this paper was submitted for publication, two additional reports that the enzyme concentration may affect the degree of inhibition have appeared: E. C. Slater reported studies with a partially purified succinoxidase system in *Biochem. J.*, 1949, **45**, 130, especially page 138, and O. H. Lowry, O. A. Bessey and E. J. Crawford have reported the effect of 2-amino-4-hydroxy-6-formylpteridine upon pterine oxidase and commented that "The extremely low dissociation of the enzyme-inhibitor complex permits a virtual titration of the enzyme." (*J. Biol. Chem.*, 1949, **180**, 399).

<sup>7</sup> Hopkins, F. G., Morgan, E. S., and Lutwak-Mann, C., *Biochem. J.*, 1938, **32**, 1829.

<sup>8</sup> Barron, E. S. G., and Kalnitsky, G., *Biochem. J.*, 1947, **41**, 346.



kinetics as carried out *in vitro*, it appears that experiments along the lines suggested by Fig. 1 and 2 would be helpful in the study of any new inhibitor. If the plot of rate against enzyme concentration is a straight line through the origin in the presence of inhibitor (*cf* malonate, Fig. 1) and if the inhibition is independent of the time of addition of substrate (in contrast to Fig. 2) then it is feasible to proceed with the determination of  $K_i$  values and tests for competitive inhibition. On the other hand, if the data are comparable to those obtained with copper, quinone, or oxalacetate, reports of inhibitor potency without showing the effect of enzyme concentration will be meaningless while the determination of  $K_i$  values and the tests for competitive inhibition by existing methods will also be of little significance.

It is from the standpoint of studies on whole animals or on tissue preparations that the results have the greatest significance. If it is established that the "irreversible" inhibitors (either pseudo-irreversible or irreversible) affect enzyme activity to an extent that depends upon the concentration of the enzyme and the concentration of inhibitor-binding compounds in the tissue, it may be anticipated that when an inhibitor (or drug or chemotherapeutic agent) is injected into an animal different tissues and tissue components will be inhibited to different extents depending on their composition. In other words, a tissue containing a small amount of a given enzyme might have its enzyme completely inactivated by an injection of an inhibitor that would inactivate only a small fraction of the enzyme in tissues containing larger amounts of enzyme and comparable amounts of other reacting components. Data which may be an illustration of this phenomenon are available in a paper by DuBois and Mangun.<sup>9</sup> Following injections of hexaethyl tetraphosphate at a level of 1 mg/kg in rats, they found that the per cent inhibitions of acetyl-choline esterase were 4.5, 22 and 100 in samples of brain, submaxillary gland and serum respectively. If these variations are

to be explained as a result of variations in the amount of cholinesterase in the 3 tissues, one would expect the enzyme content of these tissues to vary in the way that was actually observed, *viz.* the authors found relative activity values of 100, 28 and 10 in the 3 tissues from control animals. Thus the absolute amount of enzyme destroyed was very similar in the 3 tissues, *i.e.*, 4.5, 6.2 and 10.0. Injections of higher levels of inhibitor produced 100 per cent inhibition first in submaxillary gland, then in brain. These data do not prove the point that is being made here since many factors obviously affect the data in the case of whole animals; but the viewpoint stressed here may need to be included in any future examination of similar data.

The production of alloxan-diabetes<sup>10</sup> may be cited as another possible example of this type of phenomenon, with the difference that in this case specific cells have been destroyed without the demonstration of inhibition of an enzyme. Alloxan is a general SH inhibitor and it seems very unlikely that it reacts with an enzyme occurring only in the insulin-producing cells. According to our interpretation, these cells would be more vulnerable to alloxan because of smaller amounts of a vital enzyme that is more plentiful in other tissues. The specific destruction of insulin-producing cells by alloxan may serve as a prototype for cancer chemotherapy. Since the enzyme pattern in cancer tissue may conceivably include no enzymes that are not also found in normal tissues (*i.e.* if it arises by a process of enzyme deletion), the chemotherapy of cancer may require the inhibition of an enzyme that is also present in normal tissues. It should be possible to completely inhibit an enzyme present in cancer tissue in small amounts, while producing only partial inactivation of the enzyme in tissues containing it in larger amounts.

The interference phenomenon described in Fig. 2 is of considerable importance in interpreting studies on whole animals, especially since interference between reversible and ir-

<sup>9</sup> Du Bois, K. P., and Mangun, G. H., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 137.

<sup>10</sup> Lukens, F. O. W., *Physiol. Rev.*, 1948, **28**, 304.

reversible inhibitors can occur.<sup>4</sup> Koster<sup>11</sup> found that diisopropylfluorophosphate (DFP) given before physostigmine made animals more *sensitive* to the latter, while injections of the latter protected animals against doses of DFP that were several-fold greater than lethal. These data can be explained in terms of interference between the two inhibitors plus the fact that the DFP can be destroyed in the body as shown by Mazur.<sup>12</sup> In other words, physostigmine would lower the concentration of free enzyme and protect it against DFP for a time sufficient to allow for the destruction of DFP, while DFP initially would lower the effective concentration of enzyme thereby sensitizing the animal against physostigmine.

**Summary.** 1. The effect of enzyme concentration on the inhibition produced by certain inhibitors of the succinic dehydrogenase system has been experimentally determined. The inhibitors studied were malonate, itaconate,

oxalacetate, quinone and cupric ion.

2. The reaction between succinic dehydrogenase and copper or quinone was not immediate but required 30 to 40 minutes when the amount of inhibitor was just sufficient to produce complete inhibition.

3. The effect of the strengths of binding of the inhibitor with the enzyme on the per cent inhibition produced is shown to be related to the enzyme concentration.

4. An expression has been developed which relates the velocity of reaction to enzyme concentration in enzyme-inhibitor systems.

5. The results are discussed in relation to the chemotherapy of cancer. It is pointed out that the selective inhibition of an enzyme not unique to cancer tissue is theoretically possible.

The authors gratefully acknowledge the helpful suggestions of Dr. J. A. Bain, whose data on cholinesterase inhibition were made available to us prior to publication.

<sup>11</sup> Koster, R., *J. Pharmacol.*, 1946, **88**, 39.

<sup>12</sup> Mazur, A., *J. Biol. Chem.*, 1946, **164**, 271.

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## Mechanism of the Inhibition of Rat Brain Cholinesterase by Diisopropylfluorophosphate, Tetraethylpyrophosphate, and Eserine.\* (17314)

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Kinetic analysis of the inhibition of enzyme systems has generally been carried out by methods which assume a reversible combination of the inhibitor and enzyme to form an inactive complex.<sup>1-4</sup> Ordinarily, dissociation

constants for the enzyme-inhibitor complex are calculated according to the classical Michaelis-Menten treatment<sup>1</sup> from the concentration of inhibitor at which enzymatic activity is reduced 50 per cent. Goldstein<sup>5</sup> and Ackermann and Potter<sup>6†</sup> have pointed out that situations may exist when the assumptions used in the Michaelis-Menten derivation are no longer valid. Such a case arises when

\* Supported in part by grants from the Miller Epilepsy Fund, the Rockefeller Foundation, and the Research Council of the Scottish Rite Masons.

<sup>1</sup> Michaelis, L., and Menten, M. L., *Biochem. Z.*, 1913, **49**, 1333.

<sup>2</sup> Haldane, J. B. S., *Enzymes*, Longmans, Green & Co., London, 1930.

<sup>3</sup> Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.*, 1934, **56**, 658.

<sup>4</sup> Ebersole, E. R., Guttentag, C., and Wilson, P. W., *Arch. Biochem.*, 1944, **3**, 399.

<sup>5</sup> Goldstein, A., *J. Gen. Physiol.*, 1944, **27**, 529.

<sup>6</sup> Ackermann, W. W., and Potter, V. R., *Proc. Soc. Exp. Biol. and Med.*, 1949, **72**, 1.

† The author is indebted to Dr. V. R. Potter for making available a manuscript of this paper prior to publication and for much correspondence and discussion regarding its contents.

kinetics as carried out *in vitro*, it appears that experiments along the lines suggested by Fig. 1 and 2 would be helpful in the study of any new inhibitor. If the plot of rate against enzyme concentration is a straight line through the origin in the presence of inhibitor (*cf* malonate, Fig. 1) and if the inhibition is independent of the time of addition of substrate (in contrast to Fig. 2) then it is feasible to proceed with the determination of  $K_i$  values and tests for competitive inhibition. On the other hand, if the data are comparable to those obtained with copper, quinone, or oxalacetate, reports of inhibitor potency without showing the effect of enzyme concentration will be meaningless while the determination of  $K_i$  values and the tests for competitive inhibition by existing methods will also be of little significance.

It is from the standpoint of studies on whole animals or on tissue preparations that the results have the greatest significance. If it is established that the "irreversible" inhibitors (either pseudo-irreversible or irreversible) affect enzyme activity to an extent that depends upon the concentration of the enzyme and the concentration of inhibitor-binding compounds in the tissue, it may be anticipated that when an inhibitor (or drug or chemotherapeutic agent) is injected into an animal different tissues and tissue components will be inhibited to different extents depending on their composition. In other words, a tissue containing a small amount of a given enzyme might have its enzyme completely inactivated by an injection of an inhibitor that would inactivate only a small fraction of the enzyme in tissues containing larger amounts of enzyme and comparable amounts of other reacting components. Data which may be an illustration of this phenomenon are available in a paper by DuBois and Mangun.<sup>9</sup> Following injections of hexaethyl tetraphosphate at a level of 1 mg/kg in rats, they found that the per cent inhibitions of acetyl-choline esterase were 4.5, 22 and 100 in samples of brain, submaxillary gland and serum respectively. If these variations are

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TABLE I.

Effect of Order of Addition of Substrate and Inhibitor on Rat Brain Cholinesterase Activity.  
Basic conditions as in Fig. 1.

Order of addition	% inhibition	
	TEPP ( $8 \times 10^{-7}$ M)	DFP ( $4 \times 10^{-6}$ M)
Simultaneous	9	40
Substrate 10' before inhibitor	2	—
Inhibitor 30' before substrate	47	80

TEPP, when tissue concentration was plotted against rate for various concentrations of inhibitor a series of curves resulted, the control curve intercepting the x-axis at zero and the curves to which various amounts of TEPP had been added intercepting the x-axis at progressively larger values of tissue concentration. When these intercepts were plotted against the concentration of inhibitor (inset Fig. 3) a straight line through the origin was obtained. The same result was found with DFP as with TEPP, the former requiring higher concentrations; *e.g.*, the molarity at an x-intercept of 0.25 ml being  $11.5 \times 10^{-7}$  M DFP compared to  $4.5 \times 10^{-9}$  M TEPP. However, when the same procedure was applied to eserine a different result was obtained. It may be seen from Fig. 3, that in the case of eserine, a series of curves of different slopes results all intercepting the x-axis at zero in contrast to the DFP and TEPP curves.

Neither the eserine, the DFP, nor the TEPP inhibition of brain cholinesterase was reversed by cysteine as were the effects of the nitrogen mustards.<sup>7</sup> Atropine, which is a specific antidote for eserine and DFP *in vivo*, does not reverse their effects upon cholinesterase. Atropine itself does not affect the enzyme.<sup>7</sup> Eserine has been reported to protect brain cholinesterase against irreversible inactivation by DFP<sup>9</sup> and we have confirmed this finding.

**Discussion.** The values for the concentration required to give 50% inhibition of brain cholinesterase by DFP, TEPP, and eserine are of the same order of magnitude as those reported by others<sup>10-12</sup> using somewhat similar

conditions; but it is apparent from the data in this report and that of others, particularly Nachmansohn, *et al.*,<sup>13</sup> that conditions of enzyme concentration, incubation time, and order of addition of inhibitor and substrate can profoundly affect the results with the fluorophosphates.

Eserine was included in this study because it and prostigmine are the only other compounds whose potency as inhibitors approaches that of the alkylated fluoro- and pyrophosphates.<sup>14</sup> The data presented in Fig. 3 clearly show that, under the conditions employed in this study, there is a difference in the nature of the inhibitory processes. Accepting the criteria of Ackermann and Potter,<sup>6</sup> DFP and TEPP are seen to fall into that category of inhibitors where analysis by the classical methods will no longer apply. Further, the irreversibility of the enzyme-inhibitor complex is demonstrated by a method which does not involve dialysis,<sup>8</sup> dilution,<sup>13</sup> nor isolation.<sup>15</sup> In the case of eserine, on the other hand, the data of Fig. 3 show that analysis of its inhibitory action may be successfully performed by the classical methods.

On the basis of the data in Fig. 3 and other independent criteria<sup>8,13</sup> the inhibition of cholinesterase by DFP and TEPP may probably be assumed to be irreversible. We may then consider that the inhibitor "titrates" a

<sup>9</sup> Koelle, G. B., *J. Pharmacol. and Exp. Therap.*, 1946, **88**, 232.

<sup>10</sup> Adams, D. H., and Thompson, R. H. S., *Biochem. J.*, 1948, **42**, 170.

<sup>11</sup> Dubois, K. P., and Mangun, G. H., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 137.

<sup>12</sup> Webb, E. C., *Biochem. J.*, 1948, **42**, 96.

<sup>13</sup> Nachmansohn, D., Rothenberg, M. A., and Feld, E. A., *Arch. Biochem.*, 1947, **14**, 197, and *J. Biol. Chem.*, 1948, **174**, 247.

<sup>14</sup> Augustinsson, K. B., *Acta Physiol. Scandinavica*, 1948, **13**, Suppl. 52.

<sup>15</sup> Jansen, E. F., Nutting, M. D. F., and Balls, A. K., *J. Biol. Chem.*, 1949, **179**, 201.

the enzyme-inhibitor complex formation is irreversible (*i.e.*, the dissociation constant approaches zero) in which case the actual concentration of the enzyme may no longer be ignored. However, such irreversibility is not always plainly apparent and when independent evidence of such a state of affairs is lacking an investigator may analyze his data by the classical methods referred to above and arrive at values for dissociation constants that will be theoretically unsound. It is not always easy to devise unequivocal tests for irreversibility particularly when working with unpurified enzyme preparations such as serum, homogenates, minces, slices or tissue extracts. Ackermann and Potter<sup>6</sup> have recently proposed a method which offers a simple, graphical solution to this difficulty. During the course of a study of the effects of convulsant and anticonvulsant drugs on the cholinesterase system of rat brain tissue<sup>7</sup> we have had occasion to test the method of Ackermann and Potter using the newly discovered cholinesterase inhibitors diisopropylfluorophosphate and tetraethylpyrophosphate for which independent evidence for irreversibility exists<sup>8</sup> and compared their mechanism of action to that of the classical inhibitor, eserine (physostigmine).

*Experimental methods and results.* The general characteristics of the cholinesterase system in rat brain homogenates and the methods for its study have been previously described.<sup>7</sup> The specific conditions used in this investigation are given in table headings and figure legends.

The degree of inhibition by a given concentration of DFP or TEPP<sup>†</sup> is dependent upon the time of incubation of the inhibitor with the enzyme before addition of the sub-

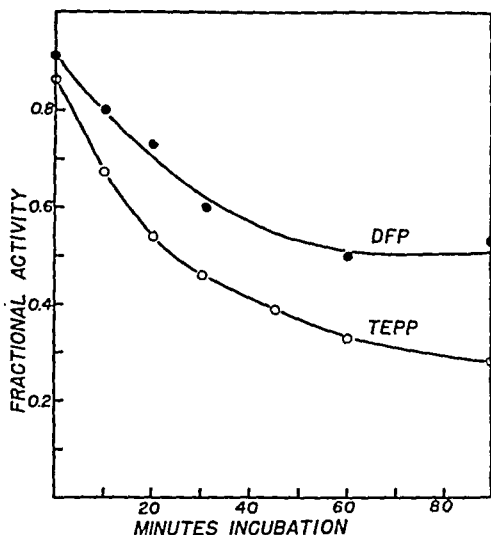


FIG. 1.

Effect of Incubation of the Enzyme with Inhibitor Before Addition of Substrate. Total volume 2.2 ml containing final concentrations as follows: 0.025 M  $\text{NaHCO}_3$ , 0.075 M KCl, 0.075 M NaCl, 0.04 M  $\text{MgCl}_2$ , 0.01 M acetylcholine-bromide (added from sidearm after incubation times indicated),  $2.3 \times 10^{-7}$  M DFP or  $8 \times 10^{-9}$  M TEPP, 25 mg (wet weight) per flask rat brain tissue added as 5% homogenate in buffer (see 7). 5%  $\text{CO}_2$ -95%  $\text{N}_2$  gas phase, temperature 37.5°C. Each point average of 2 experiments in duplicate. Activities calculated as in 7.

strate, as may be seen from the curves given in Fig. 1. In subsequent experiments we have arbitrarily adopted the time of 40 minutes incubation of the enzyme with inhibitor before addition of substrate. The enzyme may be protected from TEPP by addition of substrate simultaneous to, or before, the addition of the inhibitor (Table I). This effect is not so marked using DFP (Table I).

The inhibition of rat brain cholinesterase by DFP, TEPP and eserine under specified conditions is shown in the curves of Fig. 2 where enzyme activity is plotted against inhibitor concentration. It may be seen that the general shape of the curves is the same and without closer analysis it might be inferred that the inhibitors differed only in potency.

The results obtained using the method of Ackermann and Potter<sup>6</sup> are shown in Fig. 3. Here both tissue concentration and inhibitor concentration were varied. In the case of

<sup>7</sup> Bain, J. A., *Am. J. Physiol.*, in press.

<sup>8</sup> Bodansky, O., *Ann. N. Y. Acad. Sci.*, 1946, 47, 521.

<sup>†</sup> The abbreviations DFP—diisopropylfluorophosphate, and TEPP—tetraethylpyrophosphate, will be used throughout this paper. These compounds were obtained through the courtesy of Dr. H. E. Himwich of the Medical Division, Army Chemical Center, Edgewood, Md., and Dr. K. P. DuBois of the University of Chicago Toxicity Laboratory, Chicago, Ill.

hibitor concentration. It was shown that the kinetics of eserine-inhibition may be analyzed by classical methods which ignore enzyme concentration but the cases of the other two inhibitors may not be so treated. Cholinesterase inhibition by the fluoro- and pyrophosphates was shown to be irreversible and to depend upon enzyme concentration and time of incubation of the enzyme with the inhibitor before the addition of substrate.

The upper limit of cholinesterase concentration in rat brain was estimated to be  $1 \times 10^{-6}$  molar if structural restrictions are not assumed.

The author wishes to thank Miss Ruth Hurwitz and Mr. Richard C. Wang for valuable technical assistance.

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### Some Effects of Large Doses of Ergot Products on Rats.\* (17315)

JAY A. SMITH AND SHERWIN ZALMAN (Introduced by P. P. Foa.)

*From the Department of Physiology and Pharmacology, Chicago Medical School, Chicago, Ill.*

An examination of the literature reveals much interest in various ergot products, particularly the dihydrogenated derivatives, because of their possible usefulness in the treatment of migraine,<sup>1,2</sup> in the prevention of cyclopropane arrhythmias,<sup>3</sup> and in the treatment of hypertension.<sup>4-6</sup>

Toxicity of the dihydrogenated derivatives is less than that of the natural alkaloids;<sup>7</sup> Orth and others<sup>8</sup> show that dihydroergocornine did not produce gangrene in the tails of rats, whereas ergotamine routinely produced gangrene. They also show that pregnant female rats receiving dihydroergocornine delivered normal litters and raised them to

maturity, whereas similar rats receiving ergotamine tartrate lacked maternal instincts.

No accounts were found in the literature of experiments in which ergot products were injected over extended periods of time. Orth and others<sup>8</sup> made semiweekly injections during the gestation period of rats in doses up to 35 mg/kg of dihydroergocornine. Observations over extended periods were thought to be desirable since many patients would take the ergot product more or less regularly for years.

**Methods.** A total of 73 rats were injected subcutaneously 6 times a week for periods up to 17 weeks. This rigorous treatment contrasts with that of human therapy in which ergot products are usually injected only twice a week.

Control rats were injected 6 times a week with physiological saline.

All rats were weighed once a week.

Preliminary experiments show that doses comparable to therapeutic doses in man (for those products whose therapeutic doses have been established) produced no measurable effect on weight of rats. Therefore, it was decided to make the experiments still more rigorous by using doses comparable, on a weight basis, to those of ergotamine tartrate that produce gangrenous tails in rats. Preliminary experiments showed that a dose of about 0.5 mg/kg of ergotamine tartrate adminis-

\* This study was made possible by a grant from the Sandoz Chemical Works, Inc.; the drugs were supplied by Mr. Harry Schnizer of that company.

<sup>1</sup> Alvarez, W. C., *Gastroenterology*, 1947, 9, 754.

<sup>2</sup> Marcussen, R. M., and Wolff, H. G., *J.A.M.A.*, 1949, 139, 198.

<sup>3</sup> Orth, O. S., *Arch. internat. de pharmacodyn. et de therap.*, 1949, 78, 162.

<sup>4</sup> Kappert, A., Baumgartner, P., and Rupp, F., *Schweiz. med. Wchnschr.*, 1948, 78, 1265.

<sup>5</sup> Bluntschli, H. J., and Goetz, R. H., *South African M. J.*, 1947, 21, 382.

<sup>6</sup> Freis, E. D., Stanton, J. R., and Wilkins, R. W., *Am. J. M. Sc.*, 1948, 210, 163.

<sup>7</sup> Rothlin, E., *Bull. Schweiz. Akad. d. med. Wissensch.*, 1946-1947, 2, 249.

<sup>8</sup> Orth, O. S., Capps, R. A., and Suckle, H. M., *Fed. Proc.*, 1947, 6, 361.

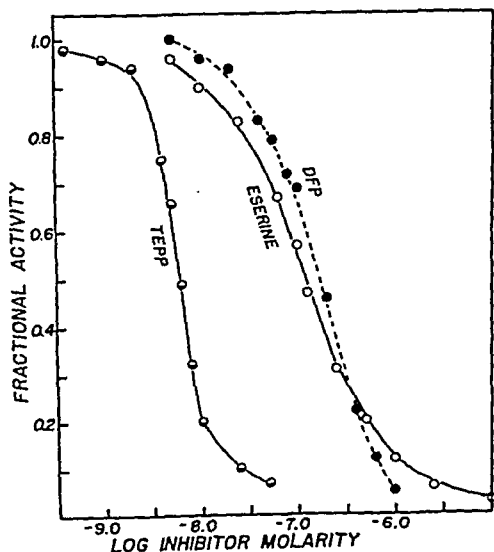


FIG. 2.

Inhibition of Rat Brain Cholinesterase by DFP, TEPP, and Eserine. Basic conditions as in Fig. 1. Inhibitors incubated with the enzyme 40 minutes before addition of substrate. Each point average of at least 2 experiments in duplicate.

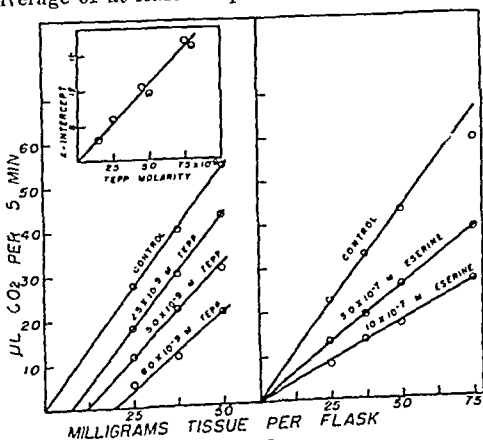


FIG. 3.

Effect of Variation in Enzyme Concentration on the Inhibition of Rat Brain Cholinesterase by TEPP and Eserine. Conditions as in Fig. 2. Inset figure: Half-solid circle - - points plotted from data given in this figure,  $\bigcirc$  - - points plotted from another duplicate experiment using different rat brain as tissue source, included to show reproducibility.

certain amount of the enzyme, evaluated by the x-intercepts in Fig. 3. If we use the most potent inhibitor, TEPP, as the limiting case, it takes of the order of  $1 \times 10^{-11}$  moles of inhibitor to titrate the enzyme in 0.25 ml of homogenate which is equivalent to 0.0125 ml

of original tissue. If we further assume that 1 mole of inhibitor combines with 1 mole of enzyme<sup>6</sup> then we may calculate that the greatest concentration of enzyme is approximately  $1 \times 10^{-9}$  moles per ml of tissue or  $1 \times 10^{-6}$  molar. The above reasoning disregards, of course, the possibility that the enzyme may be concentrated in certain regions by structural restrictions. Goldstein<sup>7</sup> has estimated that the concentration of cholinesterase in 4.5% dog serum has a maximum value of  $2 \times 10^{-8}$  M giving a value of approximately  $1 \times 10^{-7}$  M in 100% serum by direct extrapolation. We do not believe that the data in this paper justify any but the approximations indicated above because of considerable uncertainty as to the purity of the inhibitors used, the large dilutions of the inhibitor stock which are necessary and the possibility that some of the inhibitor is combined with proteins inert to the substrate employed.<sup>11</sup> The calculations do serve, however, to give an upper limit to the enzyme concentration.

**Summary.** The mechanism of inhibition of rat brain cholinesterase by the 3 most potent anti-cholinesterases, diisopropylfluorophosphate, tetraethylpyrophosphate, and eserine was studied by a method which involves variation in enzyme concentration as well as in-

§ A reasonable assumption considering that Goldstein<sup>3</sup> found 1 mole of eserine per mole of enzyme and Jansen *et al.*<sup>15</sup> have recently shown that 1 mole of DFP combines with 1 mole of chymotrypsin to form an inactive complex. We have been unable to estimate, in the case of TEPP, the number of molecules of inhibitor per molecule of enzyme by the method of Goldstein because the slope of the inhibition curve (Fig. 2) falls above his theoretically determined limits, possibly because we are dealing here with an irreversible combination.

|| This last consideration is somewhat discounted by the experiments of Mazur and Bodansky,<sup>16</sup> who added large amounts of heat-inactivated protein to a DFP-inhibited system and found no effect. However, the very process of heat inactivation may have destroyed groupings which in the native state were inert to the substrate but might not have been inert to the inhibitor.

<sup>16</sup> Mazur, A., and Bodansky, O., *J. Biol. Chem.*, 1946, 163, 261.

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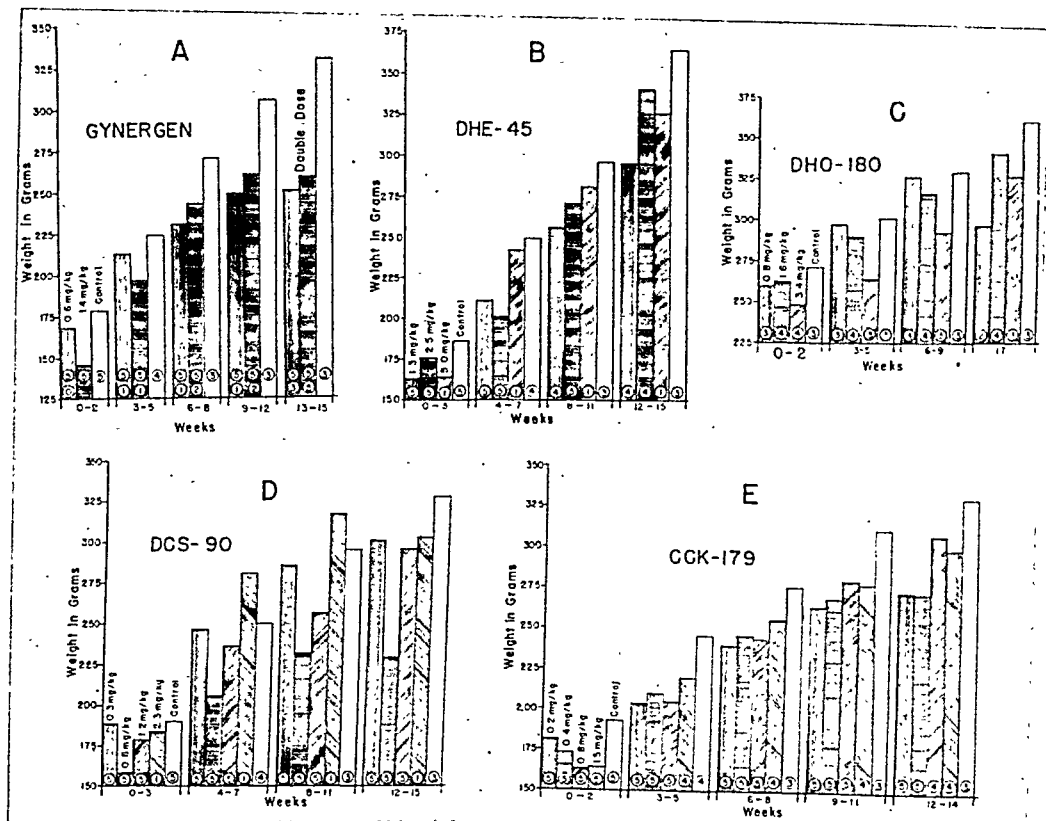


FIG. 1.

Effects of ergot products on growth rates, survival and production of gangrenous tails in rats.

A. Upper circle: Number of rats surviving the period. Lower circle: Number of rats with gangrenous tails at end of the period.

B to E, inclusive. Number encircled: Number of rats surviving the period.

tered by daily subcutaneous injection produced gangrene in about 4 weeks in about 20% of the rats. This is about 200 times the therapeutic dose in man.

The results of several weeks' weighings were averaged for simplicity in graphing. It was felt that this procedure was justified since the rate of growth of control rats was essentially linear. The results are shown in the figure.

**Results.** Ergotamine tartrate (Fig. 1, A) caused a decrease in the rate of growth in the doses employed. With doses as low as 0.6 mg/kg, one rat (20% of the rats) developed a gangrenous tail in 2 weeks. This dose was near the minimum for production of gangrene since doubling the dose caused an increase in the number of rats with gangrenous

tails within 2 days. Survival was equal to or better than controls.

Dihydroergotamine (DHE 45), dihydroergocornine (DHO 180), dihydroergocristine (DCS 90), and a mixture of dihydroergocornine, dihydroergocristine and dihydroergokryptine (CCK 179) fail to produce a consistent inhibition of growth. These materials did not produce gangrene, and the survival of rats was equal to or better than controls.

**Summary.** The effect of several dihydrogenated derivatives of ergot were studied on rate of growth, production of gangrene and survival, as compared with ergotamine tartrate. None of these materials inhibited growth consistently; none inhibited growth as much as ergotamine tartrate. None of the dihydro-

generated derivatives caused gangrene; in contrast, ergotamine produced gangrene in 80% of the rats. The survival of rats receiving ergot alkaloids or dihydrogenated ergot deriv-

atives was equal to or better than that of the controls.

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## Influence of Malononitrile upon Poliomyelitis in Mice.\* (17316)

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Recent investigations by histophysical<sup>1</sup> and histochemical<sup>2,3</sup> methods identified the basophilic component of the Nissl bodies in the nerve cells as ribonucleic acid. Chromatolysis of the Nissl substance in the late preparalytic period is the earliest cytologic change in monkeys infected with poliomyelitis virus.<sup>4</sup> While chromatolysis is a reversible process, it may lead to complete cellular necrosis. Recovery is characterized histologically by the reappearance of the Nissl substance in the surviving nerve cells. Malononitrile  $\text{CH}_2(\text{CN})_2$  increases the ribonucleic acid content, *i.e.*, the Nissl substance of the nerve cells but not of the liver and pancreas.<sup>5</sup> This prompted us to investigate the influence of malononitrile upon experimental poliomyelitis in mice.

**Materials and method.** Young, virus free mice, of the average weight of 12 to 15 g,<sup>†</sup> were infected with the Lansing strain of the poliomyelitis virus. The M.L.D. of this strain showed fluctuation during storage but was easily increased by successive mouse pas-

sages. The paralytic period, *i.e.*, the time between the appearance of the paralysis and death of the animal, was short, between a few minutes and 4 hours, even if low concentrations of the virus were used. The virus was inoculated intracerebrally, using 0.03 ml of a 10% mouse brain emulsion with an M.L.D. of  $1.3 \times 10^{-3}$ ,  $2.7 \times 10^{-5}$  and  $3.2 \times 10^{-7}$ , respectively. Malononitrile<sup>‡</sup> was administered intraperitoneally, using a 0.5 g per L. solution sterilized by passage through a Seitz filter. Since malononitrile is toxic,<sup>6</sup> its effect on normal mice was investigated.

Two types of experiments were set up. In one, the survival rate of the infected mice treated daily with malononitrile before the appearance of paralysis was compared with that of untreated controls. In the second, mice were treated with malononitrile after the onset of the paralysis and the survival time was compared with that in untreated paralyzed mice. Because of the rapid downhill course of the disease in paralyzed animals, relatively few mice could receive treatment after the onset of paralysis. Thus many animals were lost. The survival time of mice was established in such manner that the hour at which the animal was last seen alive was considered as the last hour of its life.

After the death of the animals, brain, spinal cord, liver, heart and kidneys were fixed in Carnoy's or Bouin's fluid. In addition to

\* Supported by grants from the Schering Research Fund and the Dr. Leonard H. and Louis Weissman Research Foundation.

<sup>1</sup> Landstrom, M., Caspersen, T., and Wohlfart, G., *Z. f. Mikroskop.-Anatom. Forschung*, 1941, 40, 534.

<sup>2</sup> Brachet, J., *Enzymologia*, 1941, 10, 87 and 96.

<sup>3</sup> Gersh, I., and Bodian, D., *J. Cell. Comp. Physiol.*, 1943, 21, 253.

<sup>4</sup> Bodian, D., *Bull. Johns Hopkins Hosp.*, 1948, 83, 1.

<sup>5</sup> Hyden, H., and Hartelius, H., *Acta Psychiatr. et Neurol., Suppl.*, 1948, 48, 1.

<sup>†</sup> Purchased from the Carworth Farms, N. Y.

<sup>‡</sup> Received from the Schering Corporation, Bloomfield, N. J.

<sup>6</sup> Heymans, J. F., and Masoin, P., *Arch. f. Internat. Pharmacodynam. a. Therap.*, 1897, 3, 77.

TABLE I.  
Effect of Malononitrile When Given in Preparalytic Phase.

Virus M.L.D.	No. of mice	Malononitrile given	Avg incubation time, days	Mortality, %
$1.3 \times 10^{-3}$	24	+	$37.1 \pm 13.2$	41.7
	24	0	$10.2 \pm 2.8$	100
$3.2 \times 10^{-7}$	84	+	$5.3 \pm 1.2$	97.6
	84	0	$4.4 \pm 1.3$	100

TABLE II.  
Effect of Malononitrile Given in the Paralytic Period.

Virus M.L.D.	No. of mice used	Malononitrile given	Avg survival time after onset of paralysis in hr
$2.7 \times 10^{-5}$	14	+	$43.5 \pm 8.7$
	24	0	$1.3 \pm 0.4$
$3.2 \times 10^{-7}$	22	+	$31.8 \pm 6.1$
	24	0	$0.8 \pm 0.2$

hematoxylin-eosin, the sections were stained with gallocyanin and thionine (Windle's modification<sup>7</sup>).

**Results.** The intraperitoneal administration of 6 mg malononitrile per kg body weight to normal mice produced within a few minutes rapid respiration, convulsions and death. Three mg per kg body weight was well tolerated, although a general depression following an excitation state lasted frequently for many hours.

All 24 mice which received the Lansing virus of M.L.D.  $1.3 \times 10^{-3}$  died after an incubation period of  $10.2 \pm 2.8$  days. Of 24 mice which received the same amount of the virus and 3 mg malononitrile per kg body weight the day after the infection, and, starting 6 days later, the same amount of the drug daily for 10 consecutive days, only 10, or 41.7%, died within 3 months. The average incubation time of the animals which died was  $37.1 \pm 13.2$  days (Table I).

Of 84 mice injected with a high M.L.D. ( $3.2 \times 10^{-7}$ ) all died. Of 84 animals infected with the same amount of the virus and treated with malononitrile, starting the day after infection, 82 died after an incubation time of  $5.3 \pm 1.2$  days, which does not differ significantly from that of untreated animals (*i.e.*,  $4.4 \pm 1.3$  days). Only 2 of the mice which

received malononitrile survived in this group. The period between paralysis and death in the treated animals, however, was  $17.8 \pm 1.2$  hours, as contrasted with  $0.8 \pm 0.2$  hour in untreated mice.

Of 38 mice infected with Lansing virus of M.L.D.  $2.7 \times 10^{-5}$ , 24 served as controls for the determination of the survival time after paralysis developed, while 14 mice received 3 mg malononitrile per kg body weight after paralysis appeared. The average survival time of the treated animals was  $43.5 \pm 8.7$  hours, while untreated animals lived only  $1.3 \pm 0.4$  hours after the onset of paralysis (Table II). Only 3 of the treated animals lived for less than 4 hours, while 2 survived for as long as 14 days. They showed complete recovery but died suddenly when the drug was withdrawn. In some of the other treated animals temporary partial or total disappearance of the paralysis was observed.

Of the 84 mice which received the virus in M.L.D.  $3.2 \times 10^{-7}$  concentration, 22 were treated with malononitrile after paralysis developed. Their survival time was  $31.8 \pm 6.1$  hours, as compared with  $0.8 \pm 0.2$  hour in 24 control animals. About one-third of the treated mice showed partial or complete recovery from the paralysis but died later.

The treated animals which survived for three months did not show the usual histological involvement of the central nervous sys-

<sup>7</sup> Windle, W. F., Rhines, R., and Rankin, J., *Stain Technol.*, 1943, 18, 77.

tem,<sup>8</sup> while those which died despite the treatment showed similar pathologic changes as the untreated mice except that the cytoplasmic basophilic substance of the nerve cells appeared better preserved.

**Discussion.** The described experiments indicate that malononitrile has a certain value in the treatment of mouse poliomyelitis. If lower concentrations of the virus were used, treatment in the preparalytic stage prevented death in more than one-half of the animals and prolonged the incubation period nearly threefold. While death following huge doses of the virus could not be prevented by the drug, the life span between paralysis and death was prolonged also in these cases.

When malononitrile was given after the appearance of paralysis, in about one-third of the animals significant regression of the clinical symptoms was observed. Complete recovery was observed in solitary cases but the animals died after cessation of the treatment.

There are various ways in which a chemical compound may act against the poliomyelitis virus. It may have a prophylactic effect either due to the action upon the virus before it reaches the nervous system, or it may render the nerve cells unsuitable for the multiplication of the virus. The arrangement of these experiments precludes the possibility of a

direct inactivation of the virus before it reached the nervous system. Malononitrile, however, may render the intracellular environment unsuitable for continued development of the virus by its protective effect upon the nucleic acids. It may also help to restore the damaged function by increasing the ribonucleic acid content of the attacked cells. While a direct action upon the virus within the cell is not probable, the possibility that this compound acts by interfering with an enzyme system of the host, essential for the multiplication of the virus, has to be considered.

**Summary.** Malononitrile treatment preserved the life of more than one-half of the mice infected with a low concentration of the Lansing type poliomyelitis virus, but only prolonged the life span of mice infected with large doses of the virus. Mice treated with malononitrile after the onset of the paralysis showed markedly prolonged survival time. In about one-third of the animals partial or total recovery of the paralytic symptoms was observed; the mice, however, died after cessation of the treatment.

The authors are indebted to Dr. S. O. Levinsohn from the Michael Reese Medical Research Foundation of Chicago for the virus strain, and to Mr. Chester L. Byrd, Jr., of the Hektoen Institute Virus Laboratory for his able technical help.

<sup>8</sup> Lillie, D. R., and Armstrong, C., *Publ. Health Rep.*, 1940, 55, 718.

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### Change of Muscular Excitability by Eserine, Acetone and Methyl-alcohol. (17317)

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The potentiation of acetylcholine by eserine, acetone, and methyl alcohol being known, we are interested in finding out their influence on the electrical excitability of the muscle.

The rheobase and chronaxie were determined with condenser discharge. The rectus muscle of the toad was placed in a glass muscle chamber, moistened with Ringer's solution, and observed under dissecting microscope.

After the control reading had been taken, the rectus muscle was immersed in a bath of 8 cc of Ringer's solution, bubbled with air, and treated with 10  $\gamma$  eserine for 30 minutes, or 0.05 cc acetone or methyl-alcohol for 3 minutes, and, in addition, Ringer's solution was alone used without adding any reagent for blank control. Following the treatment, both were redetermined under conditions as before.

TABLE I.  
Effect of Malononitrile When Given in Preparalytic Phase.

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$1.3 \times 10^{-3}$	24	+	$37.1 \pm 13.2$	41.7
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$3.2 \times 10^{-7}$	84	+	$5.3 \pm 1.2$	97.6
	84	0	$4.4 \pm 1.3$	100

TABLE II.  
Effect of Malononitrile Given in the Paralytic Period.

Virus M.L.D.	No. of mice used	Malononitrile given	Avg survival time after onset of paralysis in hr
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	24	0	$1.3 \pm 0.4$
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hematoxylin-eosin, the sections were stained with gallocyanin and thionine (Windle's modification<sup>7</sup>).

**Results.** The intraperitoneal administration of 6 mg malononitrile per kg body weight to normal mice produced within a few minutes rapid respiration, convulsions and death. Three mg per kg body weight was well tolerated, although a general depression following an excitation state lasted frequently for many hours.

All 24 mice which received the Lansing virus of M.L.D.  $1.3 \times 10^{-3}$  died after an incubation period of  $10.2 \pm 2.8$  days. Of 24 mice which received the same amount of the virus and 3 mg malononitrile per kg body weight the day after the infection, and, starting 6 days later, the same amount of the drug daily for 10 consecutive days, only 10, or 41.7%, died within 3 months. The average incubation time of the animals which died was  $37.1 \pm 13.2$  days (Table I).

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Of the 84 mice which received the virus in M.L.D.  $3.2 \times 10^{-7}$  concentration, 22 were treated with malononitrile after paralysis developed. Their survival time was  $31.8 \pm 6.1$  hours, as compared with  $0.8 \pm 0.2$  hour in 24 control animals. About one-third of the treated mice showed partial or complete recovery from the paralysis but died later.

The treated animals which survived for three months did not show the usual histological involvement of the central nervous sys-

<sup>7</sup> Windle, W. F., Rhines, R., and Rankin, J., *Stain Technol.*, 1943, 18, 77.

bination of acrolein with protein. If such a reaction takes place in the body, then it should follow that the configuration and chemical properties of the native protein molecule would be changed. Theoretically, protein molecules changed in this manner behave as substances foreign to the body and may act in the capacity of an antigen and cause antibody formation. Applying this principle to the pathologic physiology of burns and burn shock, one might assume that any acrolein that may be produced as a result of a burn probably combines with body protein locally or systemically. In the combined state it theoretically might be detected. Animal experimental studies were undertaken to demonstrate, if possible, by indirect evidence chemical changes in protein molecules treated with acrolein, in order to test the validity of these considerations.

*Laboratory procedure.* Freshly distilled acrolein was added to normal human and rabbit sera, in sufficient quantity so that the mixtures contained 0.5 and 1.0% of acrolein by volume. These mixtures were immediately diluted 1:5 with physiological salt solution. Sheep serum was prepared in the same manner to contain 1.5% of acrolein. The flasks were stoppered with cotton; all were then placed in a constant temperature water bath at 37°C, some for 4 days and others for 10 days to permit any excess acrolein to volatilize. At the end of this time, the contents were transferred to diaphragm rubber-stoppered bottles that were kept at refrigerator temperature (5°C). The acroleinized serum incubated at 37°C for 10 days undergoes further changes after approximately one month and will give nonspecific precipitin reactions when mixed with normal horse, rabbit, sheep or human serum. The acroleinized serum incubated at 37°C for 4 days and kept for as long as nine months was found to be free from nonspecific reactions. The microprecipitin test was used; the reactions were read within 5 minutes at room temperature. For the possible demonstration of cross reactions with formalized serum, normal human and rabbit sera were each prepared to contain 0.8% formalin gas and placed in a constant temperature water bath at 37°C for 10 days

with cotton stoppers to allow any excess formalin to volatilize. On the tenth day the formalized serum was removed and diluted 1:5 with physiologic saline solution and transferred to diaphragm rubber-stoppered bottles and stored at room temperature.

*Immunization.* Male rabbits, approximately 7 lb in weight and 5 to 6 months of age, were used. All injections were given intravenously. Acroleinized human serum incubated for 10 days was injected daily for an average of 6 days. After this a rest period of a week was allowed. The injections were repeated in this manner for a series of 4 courses of injections. The daily amount given in the first series was 0.5 ml, 1.0 ml, in the second, 2.0 ml, in the third and the final series of injections 3.0 ml. Acroleinized rabbit serum incubated for 10 days was injected in a like manner except, the first course of injections was 1.0 ml daily of 1:10 dilution; in the next 5 courses of injections a 1:5 dilution was used. The amount injected daily in the second series of injections was 1.0 ml, third 2.0 ml, fourth 3.0 ml, fifth 3.0 ml. In the sixth and final series 5.0 ml were injected every other day of the 6 day period.

Eight days after the completion of the series of injections, 3.0 ml of blood were collected from the ear vein of each of the animals receiving acroleinized serum incubated for 10 days and the antibody titer determined. Later the same day, 40.0 ml of blood were removed from the heart of each animal. To determine if bleeding would influence the antibody titer, an additional series of injections of acroleinized serum after the third series was then given as outlined above. Antibody titer increases were only slight.

Acroleinized human or rabbit serum incubated for 4 days was injected in daily single doses of 1.0 ml initially, 2.0 ml for the second, 3.0 ml for the third, and 3.0 ml for the fourth and final series of injections. Ten days following the last injection each animal was bled from the heart of approximately 50 ml.

Formalized human or rabbit serum was injected daily for 6 days and then a rest period of about a week was allowed. This was repeated for 5 courses of injections. The amount given initially at each injection was 1.0 ml,

TABLE I.  
Excitability of Rectus Muscle Increased by Eserine, Acetone, and Methyl Alcohol.

Rheobase						
Treatment	No. of observations	Before treatment		After treatment		Difference, v.
		Range, v.	Avg, v.	Range, v.	Avg, v.	
Eserine	10	.58-.71	.669 ± .014	.34-.58	.485 ± .024	.184 ± .023
Acetone	10	.65-.78	.717 ± .014	.46-.66	.583 ± .020	.134 ± .024
Methyl-alcohol	10	.65-.77	.705 ± .012	.44-.66	.569 ± .019	.136 ± .022
Ringer's solution (Blank control)	10	.92-1.34	1.106 ± .041	.90-1.33	1.085 ± .044	.021 ± .060

Chronaxie						
Treatment	No. of observations	Before treatment		After treatment		Difference, msec.
		Range, msec.	Avg, msec.	Range, msec.	Avg, msec.	
Eserine	10	1.30-7.46	3.649 ± .563	1.11- 8.14	4.007 ± .782	.358 ± .964
Acetone	10	.40-8.55	3.086 ± .710	1.54-11.0	3.859 ± .830	.773 ± 1.092
Methyl-alcohol	10	.77-6.22	4.104 ± .535	1.72- 6.75	4.810 ± .532	.706 ± .754
Ringer's solution (Blank control)	10	.87-7.96	4.064 ± .788	1.91- 9.64	5.308 ± .877	1.244 ± 1.187

It was found that rheobase was uniformly decreased by all these agents (Table I), indicating that the excitability of the muscle was definitely increased according to Chao.<sup>1</sup> Similar treatment with Ringer's solution gave no significant change.

The chronaxie showed insignificant change, and we agree with Chao<sup>1</sup> that it is not a valid measure of excitability in such a case.

*Summary.* With the threshold intensity as a criterion, the excitability of the rectus muscle is definitely increased by eserine, acetone and methyl-alcohol.

<sup>1</sup> Chao, I., *The Science Reports of National Tsing Hua Univ. Series B*, 2, 183.

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## Detection of Acrolein by Qualitative Immunochemical Analysis. (17318)

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In a previous report<sup>1</sup> it was shown that acrolein can produce a condition in experimental animals similar in its clinical and pathologic manifestations to that accepted as "shock". The problem of the detection of acrolein in blood or tissue by some specific method next presented itself. Acrolein is found to be a chemically active and highly unstable compound that does not retain its characteristics for any length of time. These

two properties alone would make it difficult to detect by the usual qualitative methods of organic analysis, especially when acrolein is present in small quantities. Secondly, as will be shown, there is experimental evidence indicating that acrolein combines directly with protein, and no longer exists in a free state. It thus becomes obvious that the usual qualitative chemical methods of detection cannot be used.

Another possible method for the detection was suggested by a postulated chemical com-

<sup>1</sup> Kamen, G. F., *Proc. Soc. Exp. Biol. and Med.*, 1943, 52, 363.

groups.

**Conclusion.** 1. These studies indicate that acrolein combines directly with some portion of the protein molecule to produce a conjugate that can be employed in the specific detection of acrolein by qualitative immunochemical methods.

2. The basic molecular pattern of human and rabbit serum protein is not changed when combined with acrolein at 37°C for 4 days. The addition of molecules of acrolein, however, does change the configuration of the protein molecule without loss of species characteristics. This is shown by the failure to form antibodies when rabbits are injected with acroleinized rabbit serum, and also by the response of the rabbits injected with acroleinized human serum by producing antibodies that reacted not only against acroleinized

human serum but normal human serum as well. These changes in the molecular configuration were specific for acrolein regardless of the basic molecular pattern used, as evidenced by cross reactions of antiacroleinized human serum with acroleinized serum of other species.

3. Foreign protein conjugates of acrolein are necessary to produce circulating antibodies that can be detected in a test tube.

Studies are now in progress on the detection of acrolein in the blood and tissues of individuals who have suffered burns.

I wish to acknowledge the helpful advice and cooperation of Dr. Alexander S. Wiener and his laboratory personnel.

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### Inhibitory Effect of Nitrogen Mustard (Bis Beta-Chloroethyl Amine) on Lesions of Experimental Serum Hypersensitiveness.\* (17319)

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Experimental serum hypersensitiveness has been studied intensively because it may serve as a useful model in promoting understanding of certain diffuse vascular diseases appearing in man. The earlier investigations of experimental hypersensitiveness were concerned primarily with morphologic characteristics of the lesions and their similarity to those of the human diseases. More recent investigations, concerned with pathogenesis, have dealt with the relationship of immunologic and vascular changes.<sup>1-4</sup> The information obtained

has led to the general conclusion that the development of humoral antibody and cutaneous hypersensitiveness is apparently related to the development of the vascular lesions. The role of immunological factors has been further investigated by determining the incidence of vascular lesions in serum-injected animals treated with drugs which may 1) prevent antigen-antibody combination or the vascular response to such combination or 2) inhibit antibody formation. Salicylates and dicumarol inhibit antigen-antibody combination *in vitro*,<sup>5,6</sup> but as yet there is no

\* This investigation was supported by a grant from the U. S. Public Health Service.

<sup>1</sup> Hawn, C. V. Z., and Janeway, C. A., *J. Exp. Med.*, 1947, 85, 571.

<sup>2</sup> Fox, R. A., and Jones, L. R., *Proc. Soc. Exp. Biol. and Med.*, 1944, 55, 294.

<sup>3</sup> Hopps, H. C., and Wissler, R. W., *J. Lab. Clin. Med.*, 1946, 31, 939.

<sup>4</sup> Sullivan, C. J., Parker, T. W., and Hibbert, R. W., *Proc. Soc. Exp. Biol. and Med.*, 1948, 67, 508.

<sup>5</sup> Coburn, A. F., and Kapp, E. M., *J. Exp. Med.*, 1943, 77, 173.

<sup>6</sup> Forman, C., Seifter, J., and Ehrlich, W. H., *J. Allergy*, in press.



TABLE I.

Antiserum	Test antigen	Antigen dilution					Titers
		1:5	1:25	1:125	1:625	1:3125	
Anti-acroleinized human serum	Acroleinized human serum	++++	++++	++++	+	—	1:125
	Normal human serum	++++	++++	++++	++	—	1:625
	Acroleinized rabbit serum	++++	++++	++	—	—	1:125
	Normal rabbit serum	—	—	—	—	—	0
	Acroleinized sheep serum	++++	++++	+++	+	—	1:125
	Normal sheep serum	—	—	—	—	—	0
	Acrolein	—	—	—	—	—	0
		—					
		—					
Anti-acroleinized rabbit serum*		—					

\* No precipitins demonstrated against any of the test antigens including acroleinized rabbit serum.

1.0 ml for the second and third, 2.0, ml for the fourth and 3.0 ml for the final series of injections. A preliminary bleeding one week after the third series of injections showed, in those rabbits receiving human serum, precipitins that reacted against normal human serum and formalized human serum, but not against normal or formalized rabbit serum. No precipitins could be demonstrated in the blood of those rabbits that received rabbit serum. The formalin concentration was then adjusted from 0.8% to 2.0% (formalin gas) and incubated at 37°C for 24 hours in sealed flasks. Two additional series of injections were then given and the rabbits bled from the heart 9 days after the last injection. The precipitin reactions were the same as before.

*Results.* Results are summarized in the table. While the rabbits injected with acroleinized rabbit serum did not respond, those injected with acroleinized human serum did produce precipitins. These precipitins reacted not only with acroleinized human serum but also with acroleinized rabbit and acroleinized sheep serum. As expected, they also reacted with normal human serum due to the formation of precipitins for normal human serum protein. That the cross reactions with acroleinized rabbit and sheep sera were due to an antibody directed against acrolein in a combined form with protein itself is indicated by the observation that the antiserum did not react with untreated rabbit or sheep serum

and also did not react with pure acrolein.

*Discussion.* The molecular configuration of rabbit serum protein is altered by acrolein but not sufficiently to act in the capacity of an antigen and cause antibody formation when injected into rabbits. Apparently the basic molecular structure of the protein characteristic of the human and rabbit species is not altered by acrolein. This is shown by the failure to form antibodies when rabbits are injected with acroleinized rabbit serum, and also by the response of the rabbits injected with acroleinized human serum by producing antibodies that reacted not only against acroleinized human serum but with also normal human serum proteins. Slight changes in the configuration of the protein molecules, however, did occur. These changes remained the same regardless of the basic molecular structure of the protein as evidenced by cross reactions of antiacroleinized human serum with acroleinized serum of other species. That the antibody was directed against the acrolein in a combined form with protein itself is indicated by the observation that the antiserum did not react with untreated rabbit or sheep serum and also did not react with pure acrolein. It would seem, therefore, that foreign protein conjugates of acrolein are necessary to produce circulating antibodies that can be detected. From the chemical nature of acrolein and protein, it may be that this linkage takes place through indole nitrogen and amino

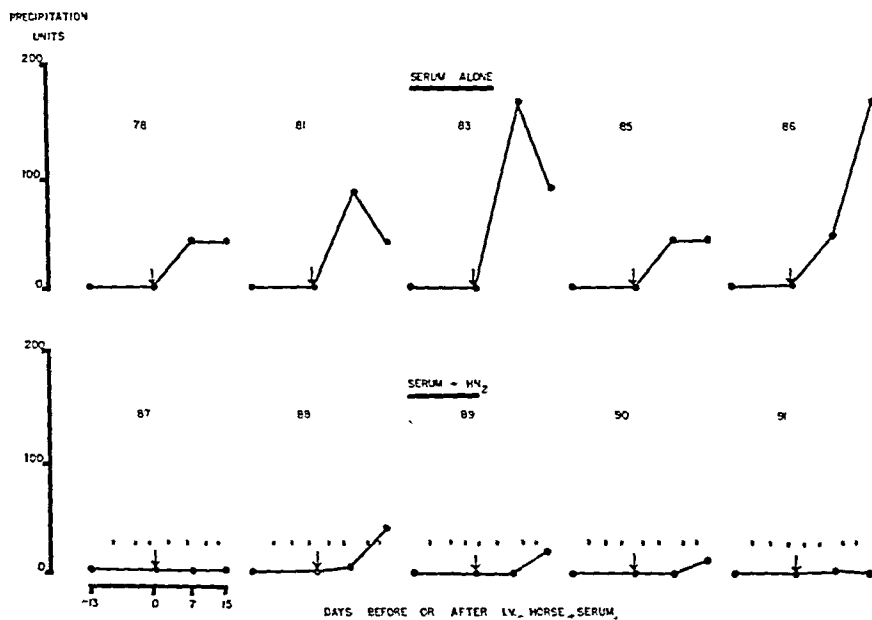
EFFECT OF  $\text{HN}_2$  ON ANTIBODY RESPONSE

FIG. 1.  
Experiment I (see text).

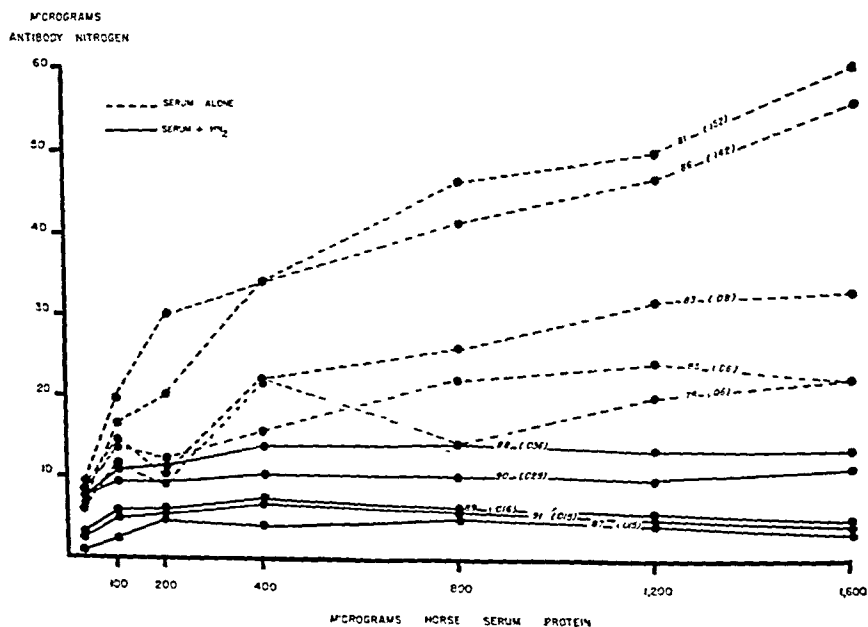
EFFECT OF  $\text{HN}_2$  ON ANTIBODY RESPONSE

FIG. 2.  
Experiment I (see text).

evidence that this effect occurs *in vivo*. That salicylates suppress the vascular lesions of serum hypersensitiveness has been reported;<sup>4,6,7</sup> the mechanism of this action has not been clarified. Since some of the cellular responses to antigen-antibody combination may be related to histamine release, antihistaminic drugs have been used in attempts to suppress the lesions of experimental serum hypersensitiveness. While such an effect has been reported,<sup>8,9</sup> our own efforts to prevent the vascular lesions with benadryl and neohetramine have been unsuccessful.<sup>10</sup>

Experiments designed to suppress antibody formation were undertaken in an attempt to establish the pathogenetic importance of antibody. Recent evidence of the importance of the lymphatic system in production of antibody suggested the use of lymphocytotoxic agents for this purpose, particularly since Hektoen and Corper<sup>11</sup> had inhibited antibody formation with sulfur mustards and recent experimental studies<sup>12,13</sup> have demonstrated similar effects by the nitrogen mustards. Furthermore, nitrogen mustard has been found capable of reducing immunity in chicken malaria.<sup>14</sup>

In the present study we have found that the nitrogen mustard, bis betachloroethyl amine ( $\text{HN}_2$ ) suppresses antibody formation, the development of cutaneous hypersensitiveness, and the incidence and severity of vascular lesions in rabbits receiving one intravenous injection of 10 ml of horse serum per kg. The results obtained suggest that the appearance

of vascular lesions bears a direct quantitative relationship to the antibody response of the experimental animal.

*Experimental.* Twenty rabbits were studied in this investigation. Ten rabbits were treated with  $\text{HN}_2$  and received intravenous serum, while an additional 10 rabbits were given serum alone.

Intravenous injections of 0.5 mg of  $\text{HN}_2/\text{kg}$  were made at 3-4 day intervals for 3 doses prior to intravenous injection of horse serum. Four additional injections at the same intervals during the subsequent 2 week period preceded sacrifice. Leucocytes were enumerated before administration of mustards, after the 5th injection (1 week after serum) and upon completion of the experiment. Qualitative serologic tests were performed by a tube precipitation procedure while quantitative antibody nitrogen (N) determinations were made colorimetrically by the technic of Heidelberger and MacPherson.<sup>15</sup> Skin tests, using 5 dilutions of horse serum, were graded according to the presence of hemorrhage and the area of edema and erythema observed 24 and 48 hours after intradermal injection of 0.2 ml.

Ten animals were observed in each of 2 experiments. In the first experiment antigen persisted in the circulation for the duration of observation in all but one of the animals. The antibody response is graphically represented in Fig. 1, in which the upper 5 curves represent antibody titers of serum controls while the lower 5 are for the  $\text{HN}_2$  treated group. It is quite evident that antibody was delayed in appearance and much reduced in amount in the  $\text{HN}_2$  treated group. This finding is even more evident in the quantitative studies of the terminal sera of all animals as indicated in Fig. 2.

The figures on each curve represent the rabbit number and, in parentheses, the calculated total antibody N content per ml of undiluted serum. Curves for the  $\text{HN}_2$  treated group are flat and the total antibody N content does not exceed .036 mg per ml, while the content of the controls varies from a minimum of .060 to .152 mg per ml.

<sup>15</sup> Heidelberger, M., and MacPherson, C. F. C., *Science*, 1943, **97**, 405; **98**, 63.

<sup>7</sup> Smull, K., Wissler, R. W., and Watson, J. M., *J. Lab. Clin. Med.*, 1948, **33**, 939.

<sup>8</sup> Kyser, F. A., McCarter, J. C., and Stengle, J., *J. Lab. Clin. Med.*, 1947, **32**, 379.

<sup>9</sup> Kyser, F. A., *Quart. Bull. Northwestern Univ. Med. School*, 1948, **22**, 256.

<sup>10</sup> Dammin, G. J., Bukantz, S. C., and Alexander, H. L., *J.A.M.A.*, 1949, **139**, 348.

<sup>11</sup> Hektoen, L., and Corper, H. J., *J. Inf. Dis.*, 1921, **28**, 279.

<sup>12</sup> Phillips, F. S., Hopkins, F. H., and Freeman, M. L. H., *J. Immunol.*, 1947, **55**, 289.

<sup>13</sup> Spurr, C. L., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 259.

<sup>14</sup> Taliaferro, W. H., and Taliaferro, L. G., *J. Inf. Dis.*, 1948, **82**, 5.

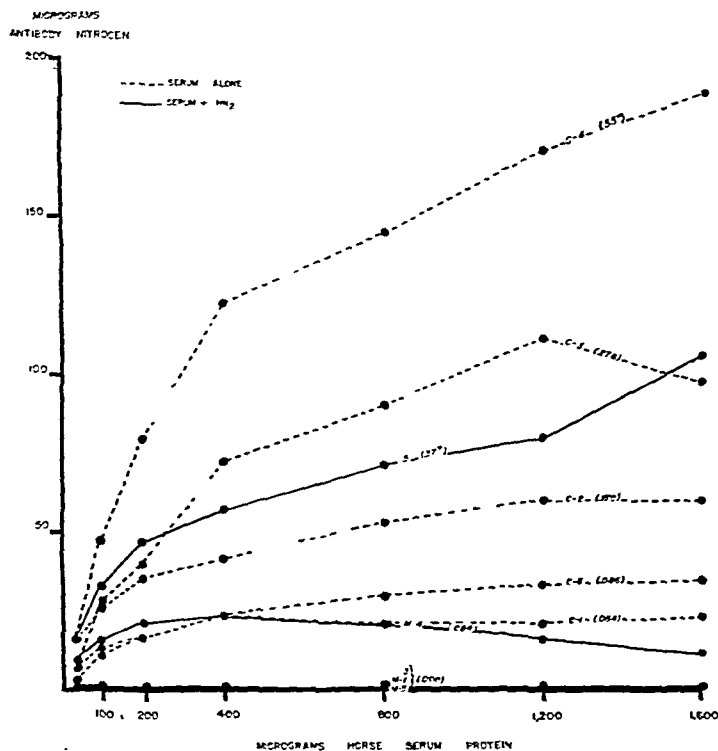
EFFECT OF  $\text{HN}_2$  ON ANTIBODY RESPONSE

FIG. 4.  
Experiment II (see text).

tions and slight vascular lesions. It was our impression that these rabbits, reacting almost exactly as untreated rabbits, had been relatively unresponsive to  $\text{HN}_2$ .

Control rabbits in this experiment exhibited a fairly wide variation in total antibody N and no evidence of leucopenia at the completion of the experiment. The severity of the vascular lesions developed by 4 of the controls was directly correlated with antibody level, the mildest lesions occurring in rabbit C-5 and the most severe in C-4. The correlation between the intensity of the Arthus reactions and the level of antibody was again imperfect, there being considerable variability in skin responsiveness.

In general, the 2 experiments reveal that the intensity of the vascular lesions correlated fairly directly with the levels of antibody N in the terminal serum samples, whether the animals had been treated with  $\text{HN}_2$  or not.

The dividing line between the appearance

of vascular lesions and their absence is at approximately .06 mg antibody N/ml. All rabbits with greater amounts of antibody developed vascular lesions, including one  $\text{HN}_2$  treated rabbit (5) of experiment II. All animals with less than .05 mg antibody N/ml failed to develop vascular lesions, with the exception of the one to which reference has already been made (M-5).

**Discussion.** The vascular lesions of experimental serum hypersensitiveness occur in animals treated in a manner known to induce the anaphylactic and Arthus type of hypersensitiveness. The importance of antibody in the latter 2 hypersensitive states has been repeatedly demonstrated<sup>16-19</sup> and it would, therefore, seem logical to conclude *a priori*

<sup>16</sup> Opie, E. L., *J. Immunol.*, 1924, **9**, 231.

<sup>17</sup> Culbertson, J. T., *J. Immunol.*, 1935, **29**, 29.

<sup>18</sup> Cannon, P. R., and Marshall, C. E., *J. Immunol.*, 1941, **40**, 127.

<sup>19</sup> Kabat, E. A., *Am. J. Med.*, 1947, **3**, 535.

TABLE I.  
Exp. I. Effect of HN<sub>2</sub> on Response to I.V. Horse Serum.

Group	Rabbit No.	Antibody† N, mg/ml	W.B.C.		Arthus‡					Vascular lesions
			Before*	After*	1:1§	1:5	1:10	1:20	1:40	
HN <sub>2</sub> treated	87	.015	12,700	2,700	++	+	+	+	0	0
	88	.036	9,050	3,200	++	+	+	+	0	0
	89	.016	10,250	1,850	++	++	+	+	0	0
	90	.029	10,200	1,400	++++	+++	+++	++	++	0
	91	.015	9,350	2,350	+++	++	+	+	0	0
Control	78	.058			++++	++++	+++	+++	++	0
	81	.15+			++++	++++	+++	+++	+++	+
	83	.08			++++	++++	+++	+++	+	+
	85	.06			++++	++++	++	++	0	+
	86	.14+			++++	++++	++	++	++	+

\* Completion of HN<sub>2</sub> Rx.  
† Terminal serum.

‡ 13-14 days after serum.  
§ Dilution of horse serum injected intradermally.

Table I summarizes the significant findings of Experiment I. The quantities of antibody N again illustrate the suppressive effect of HN<sub>2</sub> therapy. A significant leucopenia (of both granulocytes and lymphocytes) developed in all HN<sub>2</sub> treated animals. In general, Arthus reactions in the HN<sub>2</sub> treated group were less intense than those in the control group and therefore paralleled the antibody levels. Skin reactivity did not, however, correlate perfectly with antibody level as indicated by rabbit No. 90, which with 0.029 mg antibody N/ml yielded reactions equal in intensity to No. 86 with .14 mg/ml. None of the HN<sub>2</sub> treated rabbits developed vascular lesions while arterial and/or endocardial lesions of characteristic type were encountered in 4 of the 5 serum control animals. The type of arterial lesion observed is illustrated in Fig. 3.

The results of the quantitative antibody determinations on the terminal sera of Experiment II are summarized in Table II and Fig. 4. Three of the HN<sub>2</sub> treated group failed to develop any antibody despite the fact that antibody N production by the controls was considerably greater than in the first experiment. One of the 2 remaining HN<sub>2</sub> treated rabbits (5) in this experiment, however, produced amounts of antibody greater than 4 of the 5 controls and the other (M-4) more than that produced by HN<sub>2</sub> treated rabbits of the first experiment. Among the HN<sub>2</sub> treated rabbits, 3 and M-1, which had developed evident leucopenia under treatment,

developed no antibody, yielded completely negative Arthus reactions and failed to develop vascular lesions. Rabbit M-5 which died 8 days after serum injection developed evident vascular lesions, for which we have no satisfactory explanation. Rabbits 5 and M-4 showed lesser degrees of leucopenia and developed considerably more antibody than was common to the HN<sub>2</sub> treated groups; they also exhibited evident positive Arthus reac-



FIG. 3.  
A myocardial artery (from a serum control animal) exhibiting marked acute panarteritis, and well-defined medial necrosis.

# Shrinking and Swelling after Alpha Irradiation of Various Parts of Large Erythrocytes.\* (17320)

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It is well known that ionizing radiations produce hemolysis of red blood cells *in vitro*, and this seems to be associated with swelling.<sup>1</sup> Nearly all investigators have studied these phenomena with large populations of erythrocytes so that variation in behavior of the individual cells can be smoothed out to get reproducible data. As far as we know, only one previous worker has used a microbeam to irradiate portions of a single cell. Tchakhotine<sup>2</sup> irradiated egg cells with ultraviolet rays focused to a 5 micron "point" and described local damage of the membrane and underlying cytoplasm. We are aware of no similar studies with ionizing radiation.

In this study we have sacrificed the many advantages of using massive populations of erythrocytes in order to direct our attention to the microscopic changes that can be observed in individual cells before, during and after irradiation. This method has revealed a striking effect that seems to have escaped attention. Using erythrocytes of *Amphiuma* and of man, we have observed that between the time of irradiation and the well-known swelling and hemolysis there occurs a shrinkage of the cells. After total alpha irradiation of a cell the whole cell shrinks. After irradiation of a portion of a cell only the irradiated portion shrinks.

**Methods and materials.** Two methods of irradiation were used, total cell irradiation in which cells in tissue cultures on coverslips were exposed, and microbeam or partial cell irradiation in which only portions of cells were exposed. Two sources of alpha particles were

used.<sup>†</sup> For total cell irradiation the source was polonium deposited on a palladium disc 4 mm in diameter. This was mounted in a brass holder which simulated a microscope objective and could be screwed into the nose-piece of the microscope. In some experiments irradiation was administered from above by selecting a suitable field and then revolving the nosepiece to bring the sourceholder over it.<sup>3</sup> In other experiments the source-holder was mounted in the microscope condenser-holder so that cells could be irradiated from below. In the latter case, the cells were first observed with the condenser in place, then irradiated by substituting the source for the condenser, and finally observed with the condenser back in place. Alternatively, the cells could be observed continuously before, during and after irradiation by using vertical illumination without the exchanges of source and condenser.

For microbeam or partial cell irradiation a smaller source was used. The polonium was deposited on the end of a palladium wire, 0.3 mm in diameter, which was then inserted into a steel sheath made from hypodermic needle. One end of the sheath was covered with an aluminum cap, the top of which was perforated with a small hole for the exit of alpha particles. This small source was mounted on the condenser-holder. In use, a preparation was focused on the microscope stage with vertical illumination. The body tube was then racked down 20  $\mu$ , and the condenser-holder bearing the source racked up until the top of the aluminum cover with the

\* This work was done under Contract N6ori-20 with the Office of Naval Research and with the cooperation of the United States Atomic Energy Commission.

<sup>1</sup> Ting, T. P., and Zirkle, R. E., *J. Cell. and Comp. Physiol.*, 1940, **16**, 189.

<sup>2</sup> Tchakhotine, S., *Compt. rend. soc. biol.*, 1935, **120**, 714.

<sup>†</sup> The sources were furnished by the Chemistry Division of Argonne National Laboratory. We are especially indebted to Dr. W. M. Manning and Mr. P. R. Fields for their courtesy and to Mrs. Sylvia Warshaw for her services in preparing the sources.

<sup>3</sup> Zirkle, R. E., *J. Cell. and Comp. Physiol.*, 1932, **2**, 251.

TABLE II.  
Exp. II. Effect of HN<sub>2</sub> on Response to I.V. Horse Serum.

Group	Rabbit No.	Antibody N, mg/ml	W.B.C.		Arthus					Vascular lesions
			Before	After	1:5	1:10	1:20	1:40	1:80	
HN <sub>2</sub> treated	M-1	0	8,600	1,200	0	0	0	0	0	0
	M-4	.054	11,950	3,600	+++	+++	++	+	±	±
	3	0	11,500	2,400	0	0	0	0	0	0
	5	.37+	9,000	4,500	++	++	±±	±±	+	±
	M-5*	0	9,300	2,900	Not done					+
Control	C-1	.05	—	6,650	++	++	±	±	0	0
	C-2	.15	—	6,700	++	++	±±	0	0	±
	C-3	.276	—	5,750	++++	++++	+++	+++	+	+
	C-4	.55+	—	10,000	+++	++	++	±±	+	+
	C-5	.086	—	6,400	++	+	+	±	0	±

\* Died 8 days after serum I.V.

that the development of vascular lesions by the serum-injected rabbit would also be related to the development of antibody. Nevertheless, several previous studies are not in agreement with this conclusion.<sup>3,7</sup>

In the present experiments, the nitrogen mustard, bis beta-chloroethyl amine induced leucopenia and a parallel suppression of both antibody formation and vascular lesions. While suggestive, these studies, however, are not interpreted as establishing the role of anti-protein antibody in the pathogenesis of the experimental vascular lesions. The nitrogen mustards are powerful inhibitors of a number of enzyme systems and of a variety of tissue metabolic functions; some of the systems affected may possibly play a role in the pathogenesis of the vascular lesions, independent of the antibody present. It would be desirable, in defining the role of antibody, to

reproduce the lesions by passive transfer methods, to study the effects of x-rays, and to extend the present observations to include larger groups of animals before attempting to formulate recommendations regarding management of related human diseases.

*Summary.* Periodic intravenous injections of the nitrogen mustard, bis beta-chloroethyl amine, suppressed both antibody formation and the development of vascular lesions in rabbits injected intravenously with a massive dose of horse serum. The incidence of vascular lesions was directly correlated with the amount of antibody produced, since all rabbits with terminal amounts of antibody in excess of 0.06 mg nitrogen per ml exhibited vascular lesions. Cutaneous hypersensitivity failed to develop in rabbits which did not produce detectable amounts of antibody.

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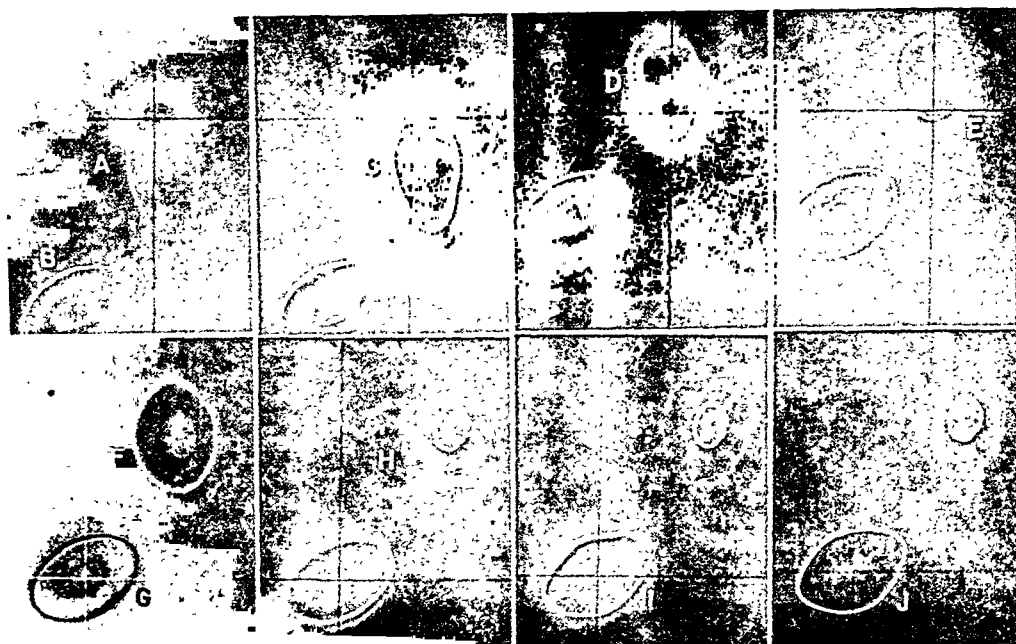


FIG. 2.

Photomicrographs of 2 *Amphiuma* erythrocytes exposed to partial cell irradiation with polonium alpha particles through a  $21.6 \mu$  hole. Each exposure lasted 90 minutes and corresponded to a dose of  $7.5 \times 10^6$  rep. (A) Cell being irradiated at one end; outline of opening through aluminum cap is also shown by focusing down  $20 \mu$  and making a second exposure; (B) normal unirradiated control. (C) 15 minutes later, one end of irradiated cell is severely shrunk; (D) the other end of the same cell is being irradiated. (E) The second end is shrinking; body of cell is swelling. (F) Swelling continues; nuclear change is evident; (G) Control cell is now being irradiated at its center. (H) First cell has hemolyzed 143 minutes after beginning of irradiation. (I) Second cell beginning to shrink, particularly on the lower side 48 minutes after beginning of irradiation; (J) Second cell begins to hemolyze 94 minutes after beginning of irradiation, (complete hemolysis, not shown in figure, occurred 120 minutes after beginning of irradiation).

it is likely that the alpha particles pass completely through the ends of the cells, whereas they do not pass completely through the cell at its center, irradiation at either end of the cell involves twice as much membrane area as is the case for irradiation at the center. It is not clear whether the longer hemolysis time of the cells irradiated at their ends is due to greater shrinkage or to the fact that the nucleus was not irradiated. Experiments are in progress to determine this point.

**Summary.** Erythrocytes from *Amphiuma* and from man were irradiated *in vitro* with polonium alpha particles (a) from an extended source (4 mm in diameter) and (b) with a small beam ( $20 \mu$  in diameter). The cells first shrank, then swelled and hemolyzed. Local irradiation of a part of a single cell resulted in shrinkage only in the irradiated portion. This was followed by swelling of the whole cell and hemolysis.

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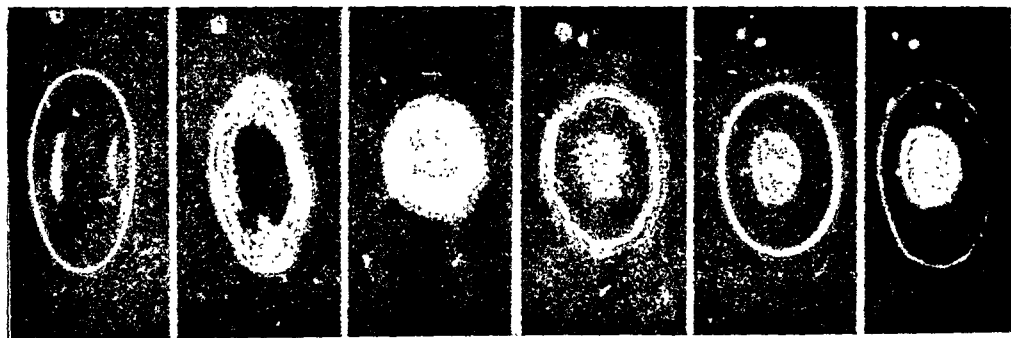


FIG. 1.

Photomicrographs of an *Amphiuma* erythrocyte showing normal cell, shrinkage, swelling, and hemolysis, after  $5 \times 10^5$  rep of alpha radiation. Vertical illumination. Length of normal cell,  $75 \mu$ .

hole came into focus. By this procedure the source was brought as close as feasible to the cells. The hole was centered with respect to ocular cross hairs. To make an exposure a piece of coverslip was withdrawn and reinserted as a shutter in the space between the aluminum cap of the sheath and the  $4 \mu$  mica slip at the bottom of the preparation.

The radiation consisted exclusively of polonium alpha particles (energy 5.3 Mev). Since these particles have a total range of only about  $35 \mu$  in tissue, the path from the source to the cells was made as short as possible to permit maximal penetration into the cells. The  $4 \text{ mm}$  source had an initial activity of  $790 \mu\text{C}$ . The distance between source and cell was  $490 \mu$  and the dose rate was calculated to be about 132,000 rep per minute. Exposures lasted from one to 50 minutes. The microbeam source had an activity of  $275 \mu\text{C}$  at the start of these experiments. The total number of particles reaching the field of irradiation was determined by direct count of those passing through the small holes used. In the case of the  $21.6 \mu$  hole, which was used most extensively, 12,500 particles emerged per minute. The area covered by the particles was determined directly. An Eastman NTA plate was substituted for a cell preparation and bombarded for one minute. A photograph of a stage micrometer was also made at the same time. Superimposing these two images gave the area of bombardment. Exposures ranged from one to 90 minutes; the average dose rate was 84,000 rep per minute.

Time of exposure was corrected for radioactive decay of the sources.

Cell preparations were made as follows. After partial caudal amputation, a drop of *Amphiuma* blood was allowed to fall upon a coverslip and was covered immediately with a mica coverslip  $3.5$  to  $4.5 \mu$  thick ( $0.7$  to  $0.8 \text{ mg per cm}^2$ ). Blood from man was drawn by skin puncture. The preparations were sealed with vaseline. Cell changes were recorded both by fast-motion cine-photomicrography and by still photomicrography. Cytological details not visible by vertical illumination were studied with phase microscopy.

**Results of Total Cell Irradiation.** Groups of irradiated erythrocytes from *Amphiuma* or man generally show the same type of response with respect to hemolysis. A typical response is shown in Fig. 1. After irradiation the cells shrink, becoming very wrinkled. Later they swell until they reach or exceed their initial size, after which they hemolyze. Cells which shrink the earliest and to the greatest extent take the longest to swell and to hemolyze.

**Results of partial cell irradiation.** Individual *Amphiuma* erythrocytes, after irradiation as described above, shrink in the irradiated portion only. However, the subsequent swelling involves the entire cell and results in hemolysis (Fig. 2).

Cells irradiated over their central portions shrink less and hemolyze significantly sooner than those irradiated at their ends. The ends of the *Amphiuma* erythrocyte are thinner than the center, which contains the nucleus. Since

TABLE II.  
Neutralization Tests with Type-3 Strain J.OI., No. 49,191.

Undiluted serum	Mouse brain suspension					
	10-1*	10-2	10-3	10-4	10-5	10-6
Normal hamster pool, 1/20/49				7/7	2/7	1/7
Type 1, T.T. hamster pool, 11/4/48		8/8	8/8	8/8	5/8	
Normal hamster pool, 6/16/47				8/8	6/8	1/8
Type 2, Fl. hamster pool, 11/8/48		8/8	8/8	8/8		
Normal mouse pool, 2/14/49			8/8	8/8		
Type 3, J.OI., mouse pool, 3/4/49	0/7	0/7				

\* Original dilutions.

Denominator indicates number of mice inoculated; numerator records number dead, paralyzed, or missing during the critical period of the test.

TABLE III.  
Effect of Type-3 Rabbit Serum on Strains of 3 Types.

Undiluted serum	Infected mouse brain suspension									
	Type 1, T.T.			Type 2, Fl.			Type 3, J.OI.			
	10-1*	10-2	10-3	10-1	10-2	10-3	10-1	10-2	10-3	10-4
Normal rabbit No. M813, 3/9/49	8/8	4/7	1/6	7/7	6/6	8/8		9/9	4/7	2/7
J.OI. immune rabbit No. M813, 5/4/49	8/8	7/7		7/7	7/7		0/6	0/7		

\* Original dilutions.

Denominator indicates number of mice inoculated; numerator records number dead, paralyzed, or missing during the critical period of the test.

One family of mice was inoculated intraperitoneally (0.05 ml.) with each dose.

One of the 1947 New York State isolations, T.T. (type 1), was selected as the representative strain for serum preparation. Each newly isolated virus was tested for neutralization by this serum. When a strain was encountered that failed to be neutralized, antiserum was prepared with it.

The first Wilmington isolation, Fl., proved to be a second type (type 2) and a 1948 New York State strain, J.OI., a third type (type 3). Cross-neutralization tests demonstrated a

distinct differentiation among the types. Representative tests have been summarized in Tables II and III.

The remaining strains when tested with these sera fell into the types indicated in Table I.

*Summary.* Among 13 virus strains which have been isolated from fecal specimens of patients with a tentative diagnosis of poliomyelitis, and which induce muscle injury in suckling mice, 3 serologic types have been encountered.

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# Serologic Differences Among Strains of the Coxsackie Group of Viruses. (17321)

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Since the isolation in suckling mice of an infectious agent from feces of 2 paralyzed children<sup>1-4</sup> a number of fecal specimens from other patients with a tentative diagnosis of poliomyelitis have been examined and 18 additional strains isolated. Some of these differ from the agent originally described in the signs of disease and the lesions they induce in suckling mice, and are being studied. The remaining 11 strains are similar to the original in causing extensive degeneration of the striated muscles of the experimental animal without damage to the central nervous system. In this group 3 serologic types have been encountered.

The localities from which the strains were obtained are indicated in Table I. The first 2 are those originally isolated. Five were obtained from fecal specimens collected in Wilmington, Del., during the 1947 epidemic of poliomyelitis and 6 from cases in 1948 in widely separated communities in New York State.

The serologic differentiation was made by the neutralization test method. Sera were prepared in mice and hamsters. Adult hamsters received living virus, in 10% hamster brain suspension, intraperitoneally in 3 or 4 weekly doses of 1 ml. Mice were given formalinized (strain T.T.) or living virus in mouse brain suspension intraperitoneally in 3 weekly series of 3 daily doses. The amounts injected increased from 0.1 to 0.5 ml. Bleedings were usually taken on the 7th or 8th day after the last dose. All mice and hamster sera were pools. Rabbits were immunized with one strain (J.OI.). A total of 35.5 to 40.5 ml. of living virus in 10% mouse brain suspension was given intravenously and intraperitoneally over a period of 36 to 46 days. Serum of only one of 3 rabbits was satisfactory. It appeared to be type- rather than species-specific.

Equal amounts of serum and infected suckling mouse brain suspension were mixed and let stand at room temperature for one hour.

TABLE I.  
Serologic Types of Strains Isolated in Suckling Mice.

Patient	Age, yr	Outbreak		Serologic type
		Year	Locality	
T.T.	9	1947	New York State	1
K.H.	3½	1947	"	1
Fl. Pool	9.5	1947	Wilmington	2
M.H.	13	1947	"	2
J.K.	8	1947	"	2
A.W.	4	1947	"	2
C.R.	12	1947	"	1
N.C.	8	1948	New York State	2
J.H.	6	1948	"	2
R.T.	4	1948	"	2
L.P.	4	1948	"	2
N.A.B.	4	1948	"	2
J.OI.	2	1948	"	3

<sup>1</sup> Dalldorf, Gilbert, and Sickles, Grace M., *Science*, 1948, 108, 61.

<sup>2</sup> Dalldorf, Gilbert, Sickles, Grace M., Plager, Hildegard, and Gifford, Rebecca, *J. Exp. Med.*,

1949, 89, 567.

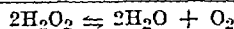
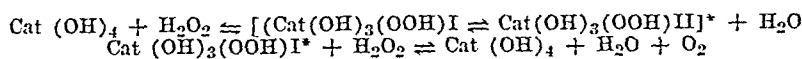
<sup>3</sup> Gifford, Rebecca, and Dalldorf, Gilbert, *Proc. Soc. Exp. Biol. and Med.*, 1949, 71, 589.

<sup>4</sup> Dalldorf, Gilbert, *Science*, in press.

TABLE II.  
Recovery of Hydrogen Peroxide by Gasometric Method.

Substrate	Added	ppm H <sub>2</sub> O <sub>2</sub> by O <sub>2</sub> evolution
Water	8.1	17.6
Amino acids	8.3	19.7
Water	16.8	28.6
Amino acids	16.8	28.5
Water	16,400	26,100
Water	32,800	52,400

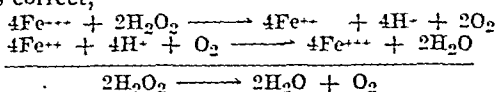
concentrations of H<sub>2</sub>O<sub>2</sub> were decomposed by acid permanganate and the oxygen evolution measured. This reaction releases one mol of O<sub>2</sub> per mol of peroxide and the concentra-



tion indicated by O<sub>2</sub> evolution always checked very closely with the initial known concentration. Thus the contradictory results are inherent in the catalase-peroxide reaction and not in the experimental method. The gas evolved by the catalase reaction was analyzed and found to be 100% oxygen. As the concentration of enzyme was decreased the total gas evolution decreased to the expected amount, one mol for each 2 mols of H<sub>2</sub>O<sub>2</sub> decomposed. Further decrease in the enzyme concentration, so that no active catalase could be detected after gas evolution ceased, gave low values for the peroxide content as would be expected from the incomplete reaction.

**Discussion.** The mechanisms postulated for the reaction between hydrogen peroxide and catalase have been discussed by Stern,<sup>6,7</sup> and Sumner,<sup>8</sup> and others. Most of the work done with catalase has been based on the rate of

reaction and not the total gas evolved.<sup>9,10</sup> It is obvious from the work by Molland<sup>11</sup> that the reaction is complex and difficult to describe by any simple set of equations. If the mechanism postulated by Keilin and Hartree<sup>10</sup> is correct,



it would appear that under the conditions of our measurements (a well buffered substrate with excess catalase) the second step does not occur. Or if the mechanism postulated by Chance<sup>12</sup> is correct,

the reaction does not go to completion under the conditions of our experiments, but stops at one of the intermediate complexes which spontaneously breaks down. It is presumed that the catalase is inactivated in yielding the 1:1 ratio of peroxide to oxygen.

**Summary.** Determinations of the peroxide content of irradiated broth using catalase in a Warburg respirometer were found to be inaccurate. In the presence of excess catalase the oxygen evolved approaches a value of twice that indicated by the accepted general equation for enzymatic decomposition of H<sub>2</sub>O<sub>2</sub>; if too little enzyme is used the reaction is slow and incomplete. The method is unsatisfactory for the quantitative determination of peroxide.

<sup>8</sup> Sumner, J. B., *Adv. Enzymol.*, 1941, **1**, 163.

<sup>9</sup> Northrop, J. H., *J. Gen. Physiol.*, 1924, **7**, 373.

<sup>10</sup> Keilin, O., and Hartree, E. F., *Proc. Roy. Soc. B.*, 1937, **124**, 397.

<sup>11</sup> Molland, J., *Acta Path. et Microbiol. Scand.*, 1947, **66**, 9.

\* Intermediate complexes formed.

<sup>12</sup> Chance, B., *Nature*, 1948, **161**, 914.

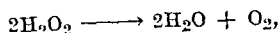
<sup>6</sup> Stern, K. G., 1942, A Symposium on Respiratory Enzymes, pp. 74-103, University of Wisconsin Press.

<sup>7</sup> Oppenheimer, C., and Stern, K. G., 1939, *Biological Oxidation*, The Hague.

## Gasometric Determination of Hydrogen Peroxide. (17322)

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We<sup>1</sup> have reported the determination of small amounts of peroxide in bacterial culture media by measurement of the oxygen evolution in a Warburg flask when a catalase preparation is added to the peroxide-containing substrate. These determinations were made because the mutagenic properties of irradiated broth have been linked with its peroxide concentration.<sup>2,3</sup> Further work on methods of peroxide determination has shown that the generally accepted overall equation for the breakdown of hydrogen peroxide by the action of catalase,



does not express the quantitative relationships that occur in the reaction as we employed it. When excess catalase was used to give the rapid completion of the reaction desired for quantitative estimation, the resulting gas output always exceeded the theoretical computed from the above equation.

**Methods.** Known concentrations of hydrogen peroxide were made by dilution of an 82% stock solution, which was checked by iodometric titration, acid permanganate titration, and the colorimetric method of Bonét-Maury.<sup>4</sup> This colorimetric method utilizes a titanium sulfate reagent which gives a yellow color in the presence of  $\text{H}_2\text{O}_2$ . A standard curve was prepared by using known concentrations of peroxide in both aqueous and amino acid substrates, and duplicate tubes were found to always agree within  $\pm 0.1\%$ . The catalase preparations used were catalase "Sarret" (furnished by Vitazyme Laboratories) and a blood preparation extracted ac-

cording to the method of Sevag.<sup>5</sup> Oxygen evolution was measured in the Warburg respirometer or, when large concentrations of peroxide were used, in a gas burette. The substrate was either water or the amino acid components of the standard synthetic medium for *Micrococcus pyogenes* prepared at 10 times the concentration used for growing the organisms.

**Experimental.** Aliquots of the amino acid solution were irradiated by a quartz ultraviolet light at various temperatures for 20 and 40 minutes and the resulting peroxide concentrations determined by the colorimetric method of Bonét-Maury and by the catalase gasometric method. Table I shows the discrepancies between these two methods. Since the colorimetric method is based on a standard curve made with known concentrations of peroxide and consistently gave accurate results when employed on known samples, it would appear that the gasometric results were in error.

As a further check of the assumed inaccuracy of the gasometric method, known concentrations of  $\text{H}_2\text{O}_2$  were added to water or to the amino acid substrate, and the peroxide concentration indicated by oxygen evolution was calculated. Table II records some typical results.

As a check on experimental methods, known

TABLE I.  
 $\text{H}_2\text{O}_2$  Content of Irradiated Amino Acid Solution.

Irradiation		ppm $\text{H}_2\text{O}_2$	
Temp.	Time, min.	Colorimetric	Gasometric
2.5°	20	4.5	6.8
2.5°	40	7.2	16.3
23°	20	9.0	18.1
23°	40	16.0	31.4
40°	20	9.5	19.8
40°	40	14.2	26.2
58°	20	9.2	19.8

<sup>5</sup> Sevag, M. G., and Mauvèg, L., *Biochem. Z.*, 1936, **288**, 41.

<sup>1</sup> Wyss, O., Clark, J. B., Haas, F., and Stone, W. S., *J. Bact.*, 1948, **56**, 51.

<sup>2</sup> Stone, W. S., Wyss, O., and Haas, F., *Proc. Nat. Acad. Sci. U.S.*, 1947, **33**, 59.

<sup>3</sup> Wyss, O., Stone, W. S., and Clark, J. B., *J. Bact.*, 1947, **54**, 767.

<sup>4</sup> Bonét-Maury, P., *Compt. rend.*, 1944, **218**, 117.

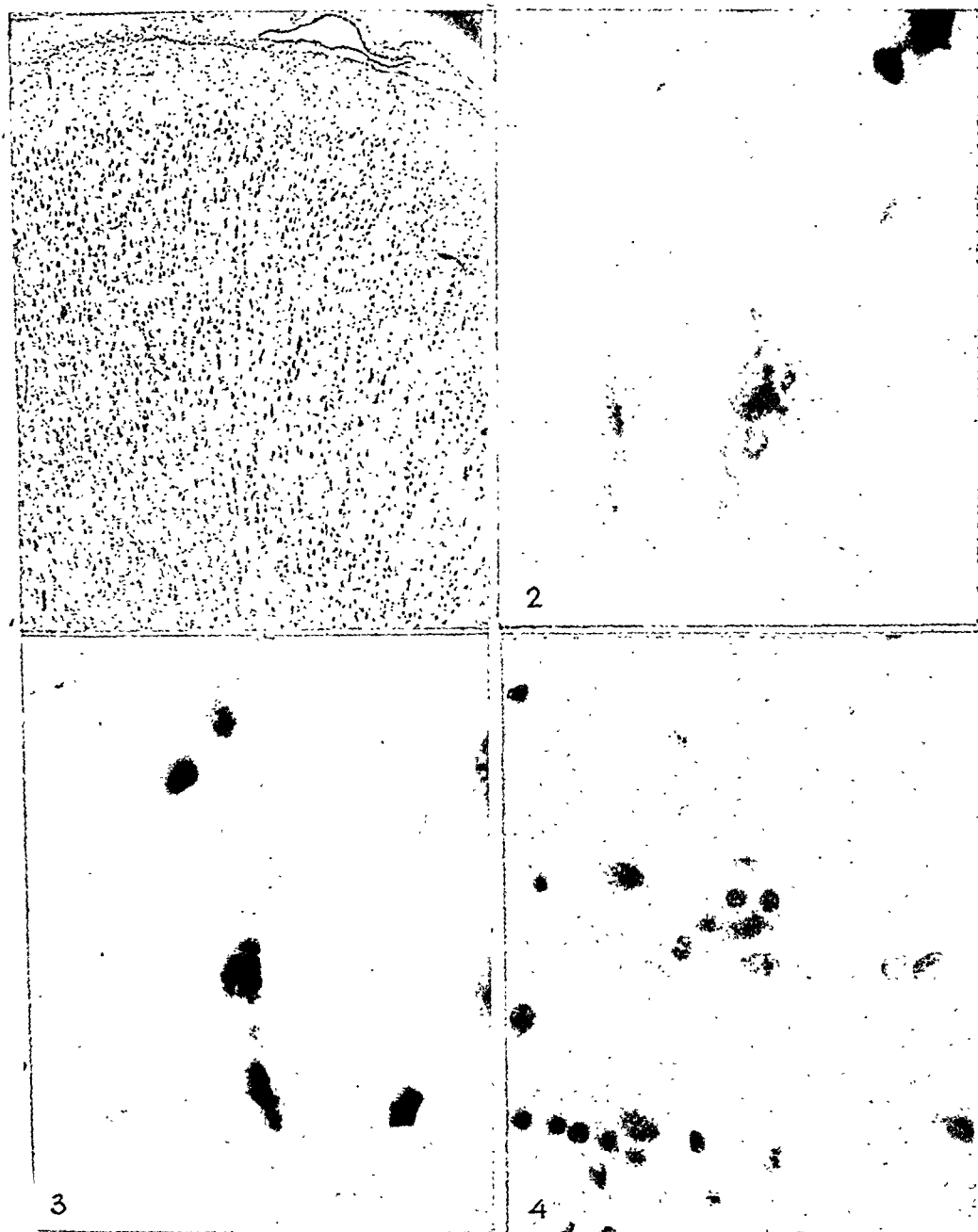


FIG. 1. Edge of small lesion in cerebral cortex 5 hours after irradiation. To the right is the area of heavy destruction, to the left that of unaffected tissue. Cresyl violet stain.

FIG. 2. Hyperchromic nerve cell of fifth cell layer of cerebral cortex. Cresyl violet stain.

FIG. 3. "Polka-dot" degeneration of cell from fifth cell layer of cerebral cortex. Cresyl violet stain.

FIG. 4. Plasma cell like changes in satellite oligodendroglia in fifth cell layer of cerebral cortex. Hematoxylin and eosin stain.

# Effects of Beta Rays on Central Nervous Tissues.\* (17323)

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The present report is made specifically to define the gross morphological changes evoked in the neurons and glial cells of the nervous system by high doses of ionizing radiations. The use of local irradiation by means of a radon applicator was made in order to study a sharp gradient of tissue reaction in the cerebral cortex. Similar technic has been described by Levin<sup>1</sup> in studies of other tissues and by Peyton<sup>2</sup> in an investigation on the effects of radium on the central nervous system. By comparing the various concentric zones of cellular alteration in a lesion the center of which showed complete destruction of cells, the pattern of progressive cytologic alteration has been made clear. In this series of experiments, the high gradient was caused both by the short trajectory of the bombarding electrons in the tissue and by the exponential operation of the law of the inverse square. For the present study, a solution of the difficult question of dosage from radon applicators will be deferred until, in a later report, the effects of dosage will be more fully discussed. Nor will attention be paid in this paper to the possible effects of the very small fraction of ionization caused by the hard gamma radiation which is delivered by the radon applicators. We have no data which would cause one to believe that the action of one type of ionizing radiation is essentially different than of another. The effects of the gamma rays in the present experiments is thought to make the gradient of exposure somewhat less steep by reason of their great tissue penetrating properties. They are, of course, also subject to the law of the inverse square and, forming a small fraction of the total ionization, seem not to complicate matters.

Cats were used in the experiments. Trephining of the calvarium over the parietal region of the cerebral cortex was performed under barbiturate anaesthesia and the dura mater carefully slit and raised. The administration of the irradiation was by means of a small glass bubble (ca 2 mm in diameter) containing emanation of radium. The heads of the cats were held in a rigid holder and the radon applicator was fastened to a 3-directional microdrive and thus directed to the surface of the cortex. The duration of the exposures varied from 5 to 32 minutes, the calculated activity of the applicators from 30 to 78 millicuries. The dura was then returned to place (in the longer experiments) and the skin carefully sutured over. Killing was by overdose of sodium pentobarbital. Where imprints of the lesions were made, the cortex was dissected out fresh and after the imprints, the material of the lesion was fixed by immersion in 10% formalin. In several of the experiments, the animal was first perfused with formalin and the lesions subsequently dissected out. Though maps of the sulci and the positions of the sites of irradiation were kept, some difficulty was had in locating those lesions which were not strongly enough irradiated to cause a visible pink spot.

*Observations.* Fig. 1 shows the edge of a typical lesion in the cerebral cortex 5 hours after exposure. To the right is the area, just visible in the photograph, of complete destruction. Towards the left the affected area borders the normal cortex. The alteration towards basophilia of the neurons and of the glia is visible. The meninges and the surface of the brain frequently are much altered at the point of contact with the applicator. In all of the marked lesions, as in Fig. 1, there was considerable breakdown of the layered structure of the cerebral cortex. The disturbance of the tissue seldom extended into the neighboring white matter. The neuronal changes in the area bordering that of complete tissue

\* Aided by a grant from The National Foundation for Infantile Paralysis.

<sup>1</sup> Levin, *Proc. Soc. Exp. Biol. and Med.*, 1924, 21, 462.

<sup>2</sup> Peyton, W., *Am. J. Cancer*, 1934, 20, 558.

they are followed through the zones of higher exposure. These consist of a marked hypertrophy of the nuclear membranes and the accumulation of visible cytoplasm. There is no sign of either cell undergoing destruction in the outer zones and the phenomenon of convergence to a common cell type is well marked. In the region of intense destruction of the neurons, these altered cells of the mesoglia show a tendency to clump around the remains of the nerve cells but signs of phagocytic activity were not made out with certainty (Fig. 4). Instead, the cytoplasm showed an absence of the foamy and detritus laden appearance of true macrophages. Examination of these altered glial cells in air-dried imprints of the lesions stained with Wright-Giemsa shows that the cells resemble, in some particulars, the brain plasma cell. The nuclei tend to be eccentric, the chromatin particles very coarse. There is a basophilic cytoplasm which resembles in its staining and texture that of the plasma cell. A perinuclear halo is, however, infrequently seen.

The changes undergone by these mesoglia cells (oligodendroglia and microglia) are similar to those of other cells of supportive tissue in inflammatory reactions. Thus the nucleolar chromatin increases in amount, the nuclear membrane hypertrophies, and both the volume and the nucleic acid content of the cytoplasm is altered upward. As is the rule in other supporting tissues, the rounding up of the cells occurs as a correlate of these processes.

In the older lesions, infiltration of haematogenous cells, first polymorphonuclear leucocytes and later lymphocytes occurs. The former species degenerate, the lymphocytes differentiate into intermediate polyblasts as described by Good.<sup>5</sup>

*Discussion.* A limiting factor in the interpretation of results such as presented here is the impossibility of ruling out the indirect effect of the irradiation on the cell components of nervous tissue by disturbance of the vascular system. Work is underway to circumvent the complication by irradiation

of cells in tissue culture. Prior to such improved data, however, the present findings are not without considerable interest.

The ability of such small radioactive sources to make restricted lesions in the nervous system has obvious uses. Not only does the small size of the lesion furnish a gradient from normal to destroyed tissue along which changes may be traced with especially good control, but bloodless destruction of certain tracts and nuclei whose vascular system is related to surface vessels may be more practical than hitherto. Details necessary for exploitation of this method in surgery must be worked out in long term experiments of this sort.

The chromophilic "sclerotic" nerve cell, so conspicuous in these preparations, has been previously studied in this laboratory (Miller,<sup>3</sup> Hartmann<sup>4</sup>) and found to be more than a fixation artifact. Production of this cell change by such means as ischemia (Tureen<sup>6</sup>), inanition, or chronic methylene blue poisoning (Näätänen<sup>7</sup>) has not been successfully duplicated in this laboratory.

Both the changes in the neurons and in the glia show the protein synthetic mechanisms of the cells to be susceptible to alteration by ionizing irradiation. The as yet undeciphered link between the genic chromatin in the nucleus, the cytoplasmic (and nucleolar) ribose nucleic acid, and physiological states associated with inflammation is apparently a target in the experiments described here. Not only in the chromophilic neuron, but particularly in the glia, is this obvious. The mesoglia elements give rise to plasma-cell like cells rather than to macrophages as would be expected in lesions of similar magnitude formed through other means. While these cells show some tendency to cluster around the neurons, phagocytic activity, as judged by the appearance of the cytoplasm, could not be postulated. As recent work on the relations of macrophages and plasma cells in experimental brain lesions has shown that

<sup>6</sup> Tureen, L. L., *Arch. Neurol. and Psychiat.*, 1936, **35**, 789.

<sup>7</sup> Näätänen, E., *Acta path. et microbiol. Scand.*, 1945, **22**, 603.

<sup>5</sup> Good, R. A., *J. Neuropath. and Exp. Neurol.*, in press.



destruction are severe and segregate into two general patterns. First is the hyperchromic reaction which leads to cells similar to those described by Miller<sup>3</sup> and Hartmann<sup>4</sup> in cerebral cortex of cats and other animals. This is characterized by a simultaneous increase in basophilia of all elements of the cell and a shrunken, ragged appearance. The second type of change observed leads to fragmentation of the nuclear chromatin and a progressive loss of nuclear membrane and cytoplasmic structure. In the area of heaviest irradiation all of the neurons of the cerebral cortex are affected. Toward the edge of the lesions it is seen the changes continue for a greater distance from the source in the large pyramidal cells of Layer 5 than in the granule cells of Layer 4. Inspection of the deeper layers closer to the center of the lesion shows that the large pyramidal cells, which seem affected by a lower dosage, also show a greater resistance to the rays in that they persist, as hyperchromic cells, in areas quite devoid of other neuron types. Further towards the center of the lesion, they, too, suffer dissolution.

The hyperchromic cells are conspicuous in Fig. 1. In Fig. 2 is shown a large pyramidal cell from Layer 5 with a long apical dendrite. All of the dendrites are deeply stained, but the cork-screw appearance mentioned by Miller and Hartmann<sup>3,4</sup> is not conspicuous. The margin of the cytoplasm is heavily vacuolated, giving the cell body a scalloped effect. The Nissl bodies are visible and discrete and of normal appearance except for the intense affinity for basic stains. The cytoplasmic matrix (the unstainable substance of Nissl), which is usually refractory to staining by most dyes, is heavily colored by the basic stain and in many of the more pycnotic cells completely occludes the finer structure of the cell. In the example seen in Fig. 2, the nucleus contains a characteristically hypertrophied nucleolus. In many instances, the nucleus has the appearance of being filled with nucleolar material. The nuclear membrane is usually heavy and covered with small densely stain-

ing plaques of chromatin. The axons are not stained.

More common than the hyperchromic alterations in the areas of heavy irradiation are those cells showing fragmentation of the nucleus and dissolution of the cytoplasm as their main features. In these cells, both the Nissl bodies and the rest of the cytoplasm have very little affinity for the stain and the nuclear changes may be followed with considerable ease. There is an increase in the amount of chromatin in the nucleus and it is seen to assume a pattern quite unlike that seen in neurons under other conditions. Heavy rounded accumulations of chromatin are formed giving the nucleus a "polka dot" appearance. In the cells least altered, as in Fig. 3, this nuclear material is of considerable bulk and a thin nuclear membrane may be made out. Other cells, apparently more affected, show loss of size of the granules and a thinning of the nuclear membrane. The nucleolus disappears. The cytoplasm of the more affected cells as well as the cell membranes are visualized only with difficulty or not at all, and near the center of the lesion remains of the neurons consist merely of an accumulation of small heavily staining granules.

The small lesions produced by these experiments are especially useful in studying the glial responses to irradiation. Across the steep gradient from normal tissue to the necrotic center of the lesion (a distance of only a few millimeters) the changes brought about by a full spectrum of adequate dosage may be observed. The fate of the astrocytes, as traced in these lesions is that of slight nuclear swelling followed by dissolution. No cytoplasm is visible in any of the stages. The nuclear material becomes finely stippled in the terminal stages, reminiscent of the dotted chromatin pattern of some of the neurons. The position in the lesion where the astrocyte may last be recognized indicates that it is more radiosensitive than either the neurons or the other glial cells.

The oligodendroglia and the microglia, whose naked nuclei may be quite well distinguished in the normal appearing periphery of the lesion show convergent changes as

<sup>3</sup> Miller, R., *Am. J. Anat.*, 1949, **84**, 201.

<sup>4</sup> Hartmann, F. P., unpublished manuscript.

TABLE I.

Granulation tissue		Titre in units/g
A. Tissue from experimental granulating canine wounds		
1. Dog. No. 1	Age of wound	
	3 days	80
	8 "	108
	14 "	208
2. Dog No. 2		
a. from center of wound,	14 days	77
b. from periphery—mostly new epithelium, 14 days		10
B. Tissue from granulating human wounds		
1. Indolent ulcer from pyoderma gangrenosum in chronic ulcerative colitis; 2 occasions		23; 30
†2. Same wound after healing had begun (chemotherapy)		170
†3. Granulating dog bite in a human (1 week)		224
4. Indolent varicose ulcer		91
5. " " "		81
†6. Granulating thoracotomy wound		564
†7. Granulating laparotomy wound		161
8. Necrotic granulations from laparotomy wound		500
†9. Infected laparotomy wound		91
†10. " " "		118
†11. " " "		159
C. Rabbit callus determinations in fractured femur		
1. 5 days		84
2. 8 "		60
3. 14 "		16

† Healthy appearing granulating tissue.

ulation tissue was therefore unexpected. The lysozyme assays on granulation tissue of man

and dog are shown in the accompanying table.

The assays were done by a viscosimetric method<sup>4</sup> on extracts prepared as previously described.<sup>3</sup>

As a result of these observations it is now apparent that high lysozyme concentrations are associated with some mesodermal cell types as well as with epithelium. Therefore, further study is warranted with regard to the role of this tissue in the production of lysozyme in ulcerative alimentary disease.

The deleterious effect of egg white lysozyme on the gastrointestinal mucosa<sup>1,3</sup> has been confirmed in our own<sup>5</sup> as well as other laboratories.<sup>6,7</sup> Furthermore, high stool titres in the absence of occult blood in the feces and with sigmoidoscopically non-ulcerated mucosa are frequently observed in chronic ulcerative colitis. These two considerations render less likely the possibility that granulation tissue is the source of the major fraction of the lysozyme titre in ulcerative alimentary disease.

<sup>3</sup> Meyer, K., Prudden, J. F., Lehman, W. L., and Steinberg, A., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 220.

<sup>4</sup> Meyer, K., and Hahnel, E., *J. Biol. Chem.*, 1946, **163**, 723.

<sup>5</sup> Prudden, J. F., Lane, N., and Meyer, K., to be published.

<sup>6</sup> Grossman, M. I., personal communication.

<sup>7</sup> Grace, W. J., Seton, D. H., Wolf, S., and Wolff, H. G., *Am. J. Med. Sci.*, 1949, **217**, 241.

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## Occurrence of Conjugase in Egg Yolks.\* (17325)

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Pteroylglutamic acid conjugases are enzymes that liberate microbiologically available PGA from more complex forms of the

vitamin and have been reported in hog kidney,<sup>1</sup> chicken pancreas,<sup>2,3</sup> rat liver<sup>4</sup> and blood.<sup>5</sup> During the course of routine PGA determina-

\* This work was supported in part by a grant from the Lederle Laboratories Division, American Cyanamid Company.

<sup>1</sup> Bird, O. D., Bressler, B., Brown, R. A., Campbell, C. J., and Emmett, A. D., *J. Biol. Chem.*, 1945, **159**, 631.

the functions of secretion of antibodies and of scavenging are sharply differentiated,<sup>5</sup> the most reasonable interpretation is that these destructive ionizing radiations have produced aberrant secretory cells, possibly analogous to the neoplastic cell in multiple myeloma.

The phenomenon of convergence of the oligodendroglia and of the microglia seen in these preparations is further evidence of the thesis supported by the senior author<sup>8,9</sup> that the microglia and the oligodendroglia are functional states of the same cell type and that both contribute to the formation of inflammatory cells in encephalitic disease. The clarity with which this process can be seen in the transition zones of the beta ray lesions makes the demonstration of similar evolution of the two species of mesoglia more obvious than any other preparation. That it is not simply a particular response to this highly unusual stimulation is substantiated by the parallelism of the changes in herpetic virus disease.

*Summary.* 1. The application of small, high-energy, applicators of radium emanation

<sup>8</sup> Campbell, B., *Anat. Rec.*, 1947, **97** (Suppl.), 7.

<sup>9</sup> Campbell, B., *J. Neuropath. and Exp. Neurol.*, 1949, **8**, 347.

to the cerebral cortex of cats produces restricted lesions in which progressive changes of the cells of that tissue may be followed from normal appearing tissue to that showing complete necrosis over a distance of several millimeters.

2. Nerve cells so affected show two types of destruction. One involves lysis of the cytoplasmic structures and degeneration of the nucleus into a small aggregation of basophilic granules. The other consists of chromophilic alteration of the cells. The pyramidal cells of layers 2, 3, and 5 seem especially susceptible to this change. The cells become shrunken, extremely heavy staining, and show hypertrophy of the nucleolus.

3. The astrocytes are the most susceptible of the various cells of the cerebral cortex. They undergo lysis in the middle zones of the lesions.

4. The mesoglia cells, oligodendroglia and microglia, are highly resistant. They show progressive changes, from the edge to the center of the lesions, which by convergence, make the two types indistinguishable. The product of these changes is a plasma-cell like structure which shows no phagocytic activity.

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## Lysozyme Content of Granulation Tissue.\* (17324)

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The highest lysozyme concentrations in the mammalian body occur in tears and in the mucosa of the antrum, pylorus, and duodenum.<sup>1</sup> The origin of these high local concentrations presumably is epithelium, *i.e.*, the tear glands and as yet undetermined cell types in the gastrointestinal mucosa. In contrast, the lysozyme titer in mesodermal tissue is low;

for example, serum averages 1 unit/cc,<sup>1</sup> and human leucocytes (from the buffy coat of normal blood) contain only 1.8 units per 5,000,000 cells.<sup>2</sup> However, human cartilage averages about 40 units/g, although this value is without doubt too low because of the difficulty in extracting this tissue. Normal human skin (including a considerable quantity of fibrous tissue) was found to have less than 1 unit/g.

The finding of high lysozyme titres in gran-

\* Supported in part by the Research Grants Division of the U. S. Public Health Service, and the Josiah Macy, Jr. Foundation.

<sup>1</sup> Meyer, K., Prudden, J. F., Lehman, W. L., and Steinberg, A., *Am. J. Med.*, 1948, **5**, 482.

<sup>2</sup> Meyer, K., in *Modern Trends in Ophthalmology*, 2:71, Paul B. Hoeber, New York, 1947.

TABLE II.  
Liberation of PGA from Egg Yolk by Different Conjugases.

Liberation by different conjugases	Free PGA	PGA liberated by egg conjugase	PGA liberated by chick pancreas conjugase	Total PGA	PGA liberated by synergistic action of egg yolk and chick pancreas conjugase	PGA liberated by egg conjugase from chick pancreas
	Micrograms of PGA per 100 g of egg yolk (fresh weight basis)					
Chicken egg yolk	10.2	21.7	25.1	81.4	24.4	-15.6
Turkey egg yolk	41.9	18.3	21.0	129.7	48.5	+0.6

were present were destroyed. Free PGA determined on such a sample amounted to 10.2  $\mu$ g per 100 g of chicken egg yolk (Tables I and II). The free PGA value for turkey egg yolk was about 4 times that of the chicken egg yolk.

The incubation of samples of egg yolk that had not been autoclaved resulted in an increase in PGA over that of the autoclaved sample described above (Tables I and II). The magnitude of this increase was about the same for both the chicken and turkey egg yolks and amounted to 21.7  $\mu$ g PGA per 100 g yolk in the chicken egg and to 18.3  $\mu$ g per 100 g in the turkey egg yolk (Table II). From these results it is apparent that egg yolk from the chickens and turkey contains a conjugase which liberates microbiologically available PGA.

When the sample was autoclaved and chick pancreas added as outlined in Flynn's procedure<sup>9</sup> this source of conjugase liberated 21.0  $\mu$ g of PGA per 100 g of chicken egg yolk and 25.1  $\mu$ g of PGA per 100 g of turkey egg yolk (Table II). These values are increases above the free PGA values and must have resulted through the action of chicken pancreas conjugase. The value obtained for chicken egg yolk where the sample was autoclaved and chick pancreas added (Table I) is in the range of that obtained by the AOAC collaborators (Flynn<sup>9</sup>) when it is converted to a dry weight basis.

The total PGA, determined by adding 20 mg of chick pancreas to the unautoclaved sample, was 81.0  $\mu$ g per 100 g for the chicken egg yolk and 129.7  $\mu$ g PGA per 100 g of turkey egg yolk. These figures are higher than that which can be calculated by adding free PGA,

PGA liberated by egg yolk conjugase and that liberated by chick pancreas conjugase (Table II). A total of these values gives 57.0 (10.2 + 21.7 + 25.1)  $\mu$ g PGA per 100 g chicken egg yolk and 81.2 (41.9 + 18.3 + 21.0)  $\mu$ g per 100 g turkey egg yolk. Thus 24.4 and 48.5  $\mu$ g PGA per 100 g egg yolk (Table II) were liberated by the synergistic action of egg yolk and chicken pancreas conjugases for the chicken and turkey egg yolks respectively. The total PGA value obtained for chicken egg yolk when converted to the dry weight basis approaches the lower value for PGA obtained by Flynn and collaborators<sup>9</sup> when dried egg yolk was assayed for PGA by the chick assay. Thus with the combined action of egg yolk conjugase and chick pancreas conjugase the value for the microbiologically available PGA approaches a similar value obtained by means of the chick assay for this vitamin.

One sample was tested in each series to determine whether egg yolk conjugase was liberating PGA from the chick pancreas. In this instance the chick pancreas solution (2 mg per ml) was autoclaved for 15 minutes at 15 lb pressure and added to the unautoclaved egg yolk. The results (Table I) show that no appreciable liberation of PGA from chick pancreas was obtained by turkey egg yolk conjugase. A slight inhibitory effect was noted on the conjugase in chicken egg yolk when autoclaved chick pancreas was added prior to incubation. The PGA liberated by egg yolk conjugase amounted to only 6.1  $\mu$ g per 100 g; whereas, 21.7  $\mu$ g PGA was liberated by egg yolk conjugase when autoclaved chick pancreas was not added. This represents a decrease of 15.6  $\mu$ g PGA per 100 g yolk in

TABLE I.  
Pteroylglutamic Acid Values Obtained by Various Treatments of Egg Yolk.\*

Treatment of sample	Autoclaved†	Not autoclaved	Autoclaved + chick pancreas	Not autoclaved + chick pancreas	Not autoclaved + autoclaved chick pancreas
Micrograms of PGA per 100 g of egg yolk (fresh weight basis)					
Chicken egg yolk	10.2	31.9	35.3	81.4	16.3
Turkey egg yolk	41.9	60.2	62.9	129.7	60.8

\* Each value in this table is the average of seven separate assays.

† All samples were incubated at 37.5° C for 24 hours.

tions in the laboratory, an indication was obtained that egg yolk also contains such a conjugase and the present study was initiated to determine if this was correct.

**Experimental.** In order to test this hypothesis, the following treatments of samples were used. In the first place the sample was autoclaved for 15 minutes at 15 lb pressure, incubated for 24 hours at 37.5°C and assayed for free PGA. In the second treatment the sample was not autoclaved but was incubated in an effort to determine if any conjugase was present in the egg yolk. In the third treatment the sample was autoclaved and 20 mg of dried chick pancreas was added prior to incubation of the sample, in order to ascertain the amount of PGA liberated by the conjugase of chick pancreas. The fourth treatment consisted of adding chick pancreas to the unautoclaved egg yolk sample in order to determine total PGA and finally 20 mg of autoclaved chick pancreas was added to a sample of egg which had not been autoclaved in order to determine if egg yolk conjugase would liberate PGA from chick pancreas. The tentative assay procedure for PGA, as devised by Flynn<sup>6</sup> for AOAC collaborators, was used in determining the microbiologically available PGA with *Lactobacillus casei* as the test organism; growth was measured by acidimetry. According to this procedure the

sample is autoclaved first and then chicken pancreas is added prior to incubation.

Chicken egg yolks used in these studies were taken from eggs laid by Single Comb White Leghorn pullets that had been in production for approximately 4 months. The turkey egg yolks were obtained from eggs laid by Broadbreasted Bronze turkey pullets that were just beginning to lay. Both the turkeys and chickens were fed practical all-mash diets and were maintained in individual laying cages with raised screen bottoms. All eggs were collected daily and placed in a refrigerator at a temperature of approximately 4.4°C. In each case the pteroylglutamic acid determination was carried out on an individual egg yolk. The egg was broken and the white was separated and discarded. A 10 g sample of the fresh yolk was blended in the Waring blender with 90 ml of distilled water. A 10 ml aliquot was pipetted into a 125 ml Erlenmeyer flask. Thirty ml of phosphate buffer (pH 7.0) and 10 ml of a chick pancreas solution, which contained 2 mg dried chick pancreas per ml, were added. The dried chick pancreas was prepared according to the method of Burkholder, McVeigh and Wilson.<sup>7</sup> The treatment of the samples prior to incubation is indicated in Table I. All samples were covered with a layer of toluene and incubated at 37.5°C for 24 hours. After incubation the samples were autoclaved for 15 minutes at 15 lb pressure, diluted to 100 ml, filtered and aliquots were taken from this stock solution for dilution purposes.

When the egg yolk sample was autoclaved for 15 minutes at 15 lb pressure and incubated it was assumed that any enzymes which

<sup>2</sup> Laskowski, M., Mims, V., and Day, P. L., *J. Biol. Chem.*, 1945, **157**, 731.

<sup>3</sup> Mims, V., and Laskowski, M., *J. Biol. Chem.*, 1945, **160**, 493.

<sup>4</sup> Sreenivasan, A., Harper, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, **177**, 117.

<sup>5</sup> Simpson, R. E., and Schweigert, B. S., *Arch. Biochem.*, 1949, **20**, 32.

<sup>6</sup> Flynn, L., Mimeographed Report to Collaborators on Folic Acid, 1948.

<sup>7</sup> Burkholder, P. R., McVeigh, I., and Wilson, K., *Arch. Biochem.*, 1945, **7**, 287.

to the changes observed in the intact animal.

*Methods.* Adult male mice of strain CBA (Strong) were employed. Littermate pairs were assigned to one of two parallel experiments. In the first, which tested the action of thyroxine in intact animals, one member of each pair was assigned at random to the treatment group while its littermate served as a control. In the second experiment, all animals were adrenalectomized, and again the pairs were divided at random into thyroxine-treated and control groups.

Until they reached the age of 11 weeks, the animals were raised on Purina Laboratory Chow and tap water. The diet was then changed to one of bread and milk to which was added whole dried Brewers' Yeast to 5% by dry weight. Both diets were fed *ad libitum*.

Treatment was begun when the animals were 13 weeks of age. The experimental animals received daily intraperitoneal injections of 15  $\gamma$  of thyroxine<sup>†</sup> in 0.1 ml of slightly alkaline distilled water. Controls received an equal volume of solvent prepared exactly as that employed for the thyroxine. The treatment period was 21 days for the intact animals; 14 days for the operated animals.

Bilateral adrenalectomy was performed in one stage via the lumbar route. Ether and light nembutal anesthesia were employed. For the first 3 post-operative days, 5% glucose and 0.9% sodium chloride were added to the drinking water of the operated animals. For the remainder of the experimental period, the drinking water contained only 0.9% sodium chloride. Operations were performed when the animals were 12 weeks of age; adrenalectomized animals were thus on the experimental diet for one week preoperatively, and were allowed a post-operative interval of one week before thyroxine therapy was instituted.

Eighteen hours after the last injection, the animals were killed by cervical dislocation and complete autopsies carried out. These were performed in a routine manner and included, in addition to the general examination, an inspection of the operative site for post-opera-

tive infection or evidence of regenerated adrenal remnants or hypertrophied accessory adrenal tissue. The following organs were dissected free of surrounding tissue and weighed on a precision torsion balance: both adrenals, both kidneys, the spleen, the thymus gland, and a sample of the peripheral lymph nodes which consisted of the two axillary nodes from each side and the two epigastric nodes. Body weights were measured on a double-beam balance at the beginning of therapy and at autopsy.

Statistical analysis involved the application of Fisher's "t-test" for small groups. In this report the word "significant" will be used only to describe differences wherein the analysis has indicated a probability value of the order of 0.01 or less.

*Results.* The results of the two experiments are collected in Table I. Since body weights do not differ significantly, absolute rather than relative values are given. For the adrenal glands, the range of the weights rather than the standard error is noted as the experimental error is thought to be greater than that due to sampling.

The administration of thyroxine to intact animals resulted in a significant increase in the weights of the spleen, peripheral lymph nodes, and kidneys. A suggestive rise in adrenal weights was observed. The weights of the thymus glands of the treated animals were less than those of their controls. While this difference amounts to almost 20% of the control values, and has a p-value of less than 0.01, the results must be regarded with caution as the sampling error is slightly less than the error generally accepted for the weights of dissected organs ( $\pm 1$  mg).

The reduced tolerance of adrenalectomized animals to thyroid hormone<sup>10</sup> was illustrated by the fact that 7 of 17 animals so treated died within 2 weeks, while all but one of the control group survived. Because of this serious loss, the experiment was terminated after a treatment period of 14 days. The results reported are those obtained for the littermate pairs in which both members survived this

<sup>†</sup> 1-thyroxine, Squibb.

<sup>10</sup> Koelsche, G. A., and Kendall, E. C., *Am. J. Physiol.*, 1935, 113, 335.

the presence of autoclaved chick pancreas (Table II).

From the results of this study maximum liberation of PGA from egg yolk occurs when egg yolk conjugase and chick pancreas conjugase act together.

*Summary.* Evidence is presented to show that chicken and turkey egg yolk contain a conjugase which liberates PGA from these materials. A synergistic action of egg yolk conjugase and chick pancreas is required in order

to obtain the total PGA in egg yolk of chickens and turkeys. The liberation of microbiologically available PGA from egg yolk by the combined action of egg yolk conjugase and chicken pancreas conjugase gives a total PGA value which approaches the value obtained by the chick assay for this vitamin. An inhibitory effect of autoclaved chick pancreas on the conjugase of chicken egg yolk is indicated by the results.

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### Effect of Thyroxine on Lymphoid Tissue Mass of Adult Male Mice.\* (17326)

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That the secretion of the thyroid gland is intimately related to the physiology of lymphoid tissue has been suggested by both clinical observation and laboratory experiment. Marine and co-workers<sup>1</sup> emphasized that peripheral lymphadenopathy, splenomegaly, and persistence of the thymus gland were detectable in many patients with Graves' disease. Several investigators<sup>2-4</sup> have reported that stimuli which cause accidental involution of the lymphoid tissue of laboratory animals may have a more pronounced or more prolonged effect in the absence of the thyroid gland.

An important link in the chain of evidence—namely that augmented thyroid secretion results in hyperplasia of lymphoid tissue—has previously evaded demonstration despite

several experimental trials.<sup>3,5</sup> One possible explanation for this failure lies in the fact that the rat was the experimental animal employed in most of these investigations. Evidence is accumulating that this animal, in the resting state, may be secreting thyroid hormone at a high rate;<sup>6</sup> thus the addition of small doses of exogenous hormone might well be without significant effect. Large doses of hormone may produce relative malnutrition and involution, rather than hyperplasia, of lymphoid tissue.<sup>7</sup> It was, therefore, considered desirable to observe the consequences of thyroxine treatment in the mouse. Profound changes in the lymphoid tissues were observed. In view of the known alterations in adrenal cortical activity following treatment with thyroid hormone,<sup>8,9</sup> a parallel experiment was conducted in adrenalectomized animals to study the possible contribution of this organ

\* Aided by a grant to Dr. Henry S. Kaplan from the National Cancer Institute, United States Public Health Service.

† Post-doctorate Research Fellow, National Cancer Institute, United States Public Health Service.

<sup>1</sup> Marine, D., Manley, O. T., and Bauman, E. J., *J. Exp. Med.*, 1924, 40, 429.

<sup>2</sup> Selye, H., *Endocrinol.*, 1937, 21, 169.

<sup>3</sup> Reinhardt, W. O., and Wainman, P., *Proc. Soc. Exp. Biol. and Med.*, 1942, 49, 257.

<sup>4</sup> White, A., and Dougherty, T. F., *Endocrinol.*, 1947, 41, 230.

<sup>5</sup> Korenchevsky, V., and Hall, K., *Biochem. J.*, 1941, 35, 726.

<sup>6</sup> Meites, J., and Chandraseker, B., *Endocrinol.*, 1949, 44, 368.

<sup>7</sup> Andreasen, E., *Acta path. et microbiol. Scand.*, 1939, 15, 259.

<sup>8</sup> Lowenstein, B. E., and Zwemer, R. L., *Endocrinol.*, 1943, 33, 36.

<sup>9</sup> Deane, H. W., and Greep, R. O., *Endocrinol.*, 1947, 41, 243.

related to an increased rate of protein metabolism. It is perhaps significant that adrenal cortical hypertrophy, well known to occur under thyroid treatment, is also thought to be a response to an increased rate of protein utilization.<sup>17</sup> In addition, evidence has recently been presented that lymphoid tissue may represent an important source of metabolic protein<sup>18</sup>—a source limited in volume, but of extreme lability. While the present experiments record only gross structural alterations, and the possible contribution of these changes to the physiology of the organism

must remain in the realm of speculation, it is suggested that an increased rate of nitrogen metabolism may well represent the common denominator underlying the many changes noted in this investigation.

*Summary.* The administration of thyroxine to adult male mice resulted in an increase in the organ weights of the kidneys, spleen, and peripheral lymph nodes. Similar treatment of adrenalectomized animals resulted in an increase in the weights of the spleen and peripheral lymph nodes over and above that which followed adrenalectomy alone. It is concluded that thyroxine produces an increase of lymphoid tissue mass which is independent of the rate of body growth and of the level of adrenal cortical activity.

<sup>16</sup> Koehakian, D. C., in *Recent Progress in Hormone Research*, Vol. 1, New York, Academic Press, Inc., 1947, p. 177.

<sup>17</sup> Tepperman, J., Engel, F. L., and Long, C. N. H., *Endocrinol.*, 1943, **32**, 373.

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## Failure of Aureomycin in the Treatment of Experimental Tuberculosis. (17327)

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The failure of streptomycin, p-aminosalicylic acid and the sulfone compounds to prove ideal agents for the treatment of tuberculosis has stimulated the testing of many new agents. Because of the remarkably wide anti-microbial spectrum of aureomycin, it seemed worthwhile to study its effect upon experimental tuberculosis.

Guinea pigs weighing 400 to 500 G each were inoculated subcutaneously with 0.02 mg tubercle bacilli from a recently isolated, virulent, streptomycin-sensitive human strain. After 28 days, the animals were divided into two groups. The 12 animals in Group A were treated with daily subcutaneous injection of 5 mg aureomycin hydrochloride, 6 days a week for 6 weeks. The 11 animals in Group

B served as infected, untreated controls.

A third group, Group C, consisted of 6 uninfected guinea pigs given aureomycin in the same dosage as Group A, in order to observe possible toxic effects of the drug.

Animals were autopsied as they died, and those surviving after 6 weeks of treatment or 10 weeks after infection were sacrificed and autopsied.

In Table I, it can be seen that mortality was higher in the treated than in the untreated group. Both treated infected and uninfected groups showed marked weight loss, and one of the uninfected animals died during treatment.

All the infected animals showed widespread tuberculous involvement of the regional lymph nodes surrounding the injection site, the liver, spleen, and lungs. Gross and microscopic examination failed to show any appreciable difference between the treated and untreated animals. Acid fast bacilli were demonstrated

\* The author acknowledges the kind assistance of Ralph Knutti, M.D., who reviewed the microscopic sections. Aureomycin was supplied by Lederle Laboratories Division, American Cyanamid Company.



TABLE I.  
Body Weights and Organ Weights of Adult Male Mice Treated with Thyroxine.

Group	No. of mice	Body wt (g)		Organ wt (mg)				
		Initial	Final	Spleen	Lymph nodes	Thymus	Kidneys	Adrenals
Intact Control	14	28.3 ±0.5	29.7 ±0.5	103 ±3.4	32 ±1.2	27 ±0.9	470 ±9.6	4 (3-5)†
Intact Thyroxine*	14	28.1 ±0.5	29.5 ±0.5	170 ±8.2	53 ±4.1	22 ±0.9	586 ±13.3	5 (4-6)†
Adrenalectomized Control	9	27.4 ±0.6	28.3 ±0.6	161 ±5.3	47 ±1.8	45 ±1.2	441 ±10.8	—
Adrenalectomized Thyroxinet	9	27.3 ±0.6	28.2 ±0.7	224 ±5.6	61 ±3.2	53 ±3.5	500 ±27.6	—

\* 15  $\gamma$  per day for 21 days.

† 15  $\gamma$  per day for 14 days.

‡ Range of values. Other values are means and standard error by

$$\frac{\sqrt{\Sigma d^2}}{\sqrt{n(n-1)}}$$

length of time. These results illustrate the well-known effects of adrenalectomy on lymphoid tissues. In addition the weights of the spleen and lymph nodes of the thyroxine-treated animals are significantly higher than those of their controls. The thymus weights showed an increase of limited significance ( $p = <0.05$ ). The shorter course of thyroxine treatment employed for this group did not result in a significant change in kidney weights ( $p = >0.05$ ). Body weight values demonstrate that the dietary regimen adequately maintained the operated animals; and the stability of these values indicates that the animals are well past the period of most active growth (4 to 8 weeks).<sup>11</sup>

**Discussion.** While quantitative histologic studies were not performed, microscopic examination suggested that much of the increase in lymphoid tissue mass was due to hyperplasia of these organs. In response to either thyroxine or adrenalectomy, the spleen and peripheral nodes registered an increase in weight of over 50%. The two treatments appeared to be additive and together resulted in a doubling of the weights of these organs. That this increase following thyroxine treatment occurred in animals showing essentially stable body weights indicates that the effect

of this treatment was quite distinct from any that might result from a change in rate of growth.<sup>12</sup> Furthermore, exhaustion of the adrenal cortex<sup>9</sup> does not appear to have contributed to this effect, since treatment of adrenalectomized animals with thyroxine resulted in an increased weight of the spleen and peripheral nodes over and above that following adrenalectomy alone. If anything, the data for the adrenal and thymic weights of the first experiment suggest augmented adrenal cortical activity during the period studied. The slight decrease in thymic weights of intact animals treated with thyroxine appears not to have occurred in the adrenalectomized animals. This behavior of the thymus, in contrast to the other lymphoid tissues studied, is consistent with its greater sensitivity to the action of adrenotrophic hormone.<sup>13,14</sup>

An increase in kidney weight has been previously shown to follow thyroid treatment in rats<sup>15</sup> and testosterone treatment in mice.<sup>16</sup> In both instances, the renal hypertrophy was

<sup>12</sup> Koger, M., Hurst, V., and Turner, C. W., *Endocrinol.*, 1942, **32**, 237.

<sup>13</sup> Dougherty, T. F., and White, A., *Proc. Soc. Exp. Biol. and Med.*, 1943, **53**, 132.

<sup>14</sup> Simpson, M. E., Li, C. H., Reinhardt, W. O., and Evans, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1943, **54**, 135.

<sup>15</sup> MacKay, E. M., and MacKay, L. L., *J. Nutrition*, 1931, **4**, 33.

<sup>11</sup> Morris, H. P., *J. Nat. Cancer Inst.*, 1944, **5**, 115.

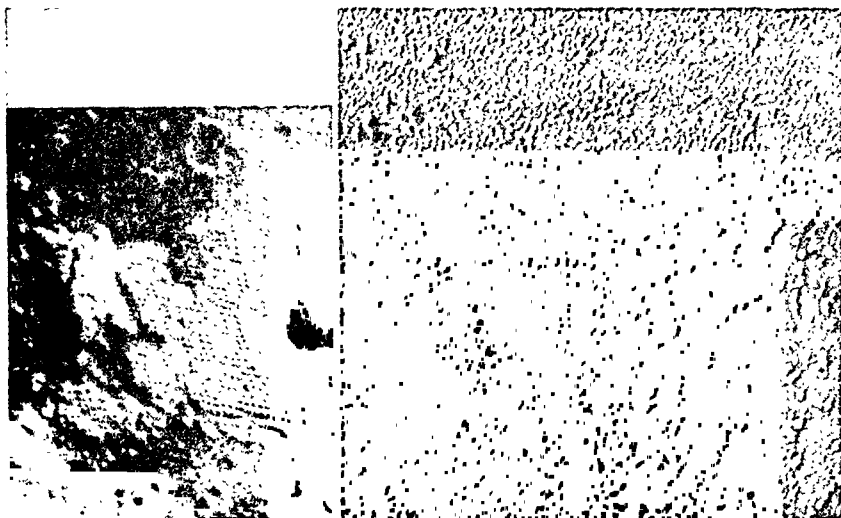


FIG. 1A. Electron micrograph of virus-like particles from intranuclear inclusion body papilloma. Note that particles are in crystalline-like array as well as free. Shadow cast with chromium, angle 1:7. Magnification 18,700  $\times$ .

FIG. 1B. Same from another patient.

tern. Five such lesions were studied by electron microscopy. Four were obtained from the hands of children whose ages ranged from 2 to 12. These lesions differed from common warts in having a more pearly base and an erythematous halo, and were separated easily and completely from the underlying tissue with a curette. The fifth was a plantar wart obtained from a child 7 years old. It differed from the typical plantar wart in that there was no noteworthy keratotic tor-

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and the resuspended sediment were placed on collodion mounts for electron microscopy. These were shadow cast with chromium at an angle of 1:7.

Examination with the electron microscope (RCA type EMU) revealed spherical particles most abundantly in the 6,000 r.p.m. sediment of the suspension from the papillomas showing intranuclear inclusion bodies. These particles were frequently arranged in crystalline-like clusters or layers with an average diameter of 52 m $\mu$  and a range of 50 to 53 m $\mu$  (Fig. 1). Such an arrangement resembles previously noted for crystalline plant (Price, Williams, Wyckoff<sup>1</sup>). When particles were not in crystal-like array, they had a diameter of 68 m $\mu$  with a range

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C., Wyckoff, R.

TABLE I.  
Effect of Aureomycin on Weight and Survival of Tuberculous Guinea Pigs.

Group	Avg wt start, g	Avg wt start treatment, g	Avg wt death, g	No. animals start	No. animals surviving 10 wks
A. Infected, aureomycin treated	501	556	440	12	5
B. Infected, untreated	471	529	552	11	10
C. Uninfected aureomycin treated	465	652	578	6	5

in the microscopic sections of tuberculous tissue from the 5 animals which survived the full 6 weeks of treatment, and tubercle bacilli were readily cultured from the lung in one of these animals.

All the animals treated with aureomycin showed definite evidence of local drug toxicity. There was marked fibrosis of the anterior abdominal wall, where the injections were given, and there were extensive peritoneal adhesions, which, in a few animals, constricted and partially obstructed loops of small intestine. This mechanical obstruction may have accounted for the weight loss in some of the treated animals. In 6 of the treated animals, intercellular material that stained blue with hematoxylin was observed in the liver, spleen, or renal medulla. In 2 of the treated animals, focal hepatitis and hepatic necrosis were observed.

The *in vitro* aureomycin sensitivity of the original organism used, when grown in a modified Dubos liquid medium, was 100 µg/cc. The sensitivity of the strain recovered from the guinea pig after 6 weeks of aureomycin treatment was unchanged.

These results confirm the findings of Steenken and Wolinsky,<sup>1</sup> who failed to influence favorably guinea pig tuberculosis using a considerably lower dosage of aureomycin. It is shown here that aureomycin given in parenteral per kg dosage approximately 10 times the maximum used for other infections in man fails to influence favorably the course of guinea pig tuberculosis, and, indeed, exhibits definite toxicity.

<sup>1</sup> Steenken, W., Jr., and Wolinsky, E., *Am. Rev. Tuberc.*, 1949, **59**, 221.

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### "Crystalline" Virus-Like Particles from Skin Papillomas Characterized by Intranuclear Inclusion Bodies.\* (17328)

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(With technical assistance of Ruth Peabody.)

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This is a report of the observation with the electron microscope of virus-like bodies that have been obtained from skin papillomas. These papillomas are characterized by the presence of intranuclear inclusion bodies, and the elementary bodies obtained from them tend to be arranged in a crystalline-like pat-

\* Supported by grants from the Fluid Research Fund of the Yale University School of Medicine and from the National Cancer Institute of the National Institute of Health, U. S. Public Health Service.

<sup>†</sup> National Research Council Fellow in Medical Sciences.



FIG. 1A. Electron micrograph of virus-like particles from intranuclear inclusion body papilloma. Note that particles are in crystalline-like array as well as free. Shadow cast with chromium, angle 1:7. Magnification 18,700  $\times$ .

FIG. 1B. Same from another patient.

tern. Five such lesions were studied by electron microscopy. Four were obtained from the hands of children whose ages ranged from 2 to 12. These lesions differed from common warts in having a more pearly base and an erythematous halo, and were separated easily and completely from the underlying tissue with a curette. The fifth was a plantar wart obtained from a child 7 years old. It differed from the typical plantar wart in that there was no noteworthy hard hyperkeratotic top and very little surrounding callus. After a circular incision was made around the periphery this lesion also was easily and completely separated from the underlying tissue with a curette. Common warts from 9 patients, *molluscum contagiosum* lesions from 2 patients and one example of normal skin were similarly studied.

The preparation of the material was the same in all instances. Half of each lesion was fixed in formalin for tissue sections while the remainder was promptly ground with alundum and distilled water and subjected to clarifying centrifugation of the supernatant fluid at 2,000 r.p.m. for 5 minutes followed by centrifugation of the resulting supernatant fluid at 6,000 r.p.m. for 15 to 45 minutes. Small drops of the 6,000 r.p.m. supernatant fluid

and the resuspended sediment were placed on collodion mounts for electron microscopy. These were shadow cast with chromium at an angle of 1:7.

Examination with the electron microscope (RCA type EMU) revealed spherical particles most abundantly in the 6,000 r.p.m. sediment of the suspension from the papillomas showing intranuclear inclusion bodies. These particles were frequently arranged in crystalline-like clusters or layers with an average diameter of 52  $m\mu$  and a range of 50 to 53  $m\mu$  (Fig. 1). Such an arrangement resembles that previously noted for crystalline plant viruses (Price, Williams, Wyckoff<sup>1</sup>). When these particles were not in crystal-like array, they averaged 68  $m\mu$  in diameter with a range of 56 to 80  $m\mu$ .

Control preparations of the *molluscum contagiosum* lesions revealed the characteristic brick-shaped elementary bodies previously noted by Boswell<sup>2</sup> (Fig. 2). These had an average length of 330  $m\mu$  with a range of 190 to 250  $m\mu$ . They were found most abundantly in the 6,000 r.p.m. sediment.

<sup>1</sup> Price, W. C., Williams, R. C., Wyckoff, R. W. G., *Science*, 1945, 102, 277.

<sup>2</sup> Boswell, F. W., *Brit. J. Exp. Path.*, 1947, 128, 253.



FIG. 2.

Electron micrograph of brick-shaped elementary bodies from molluscum contagiosum lesions. Magnification  $35,000\times$ .

These were also seen with the electron microscope in preparations made simply by touching the molluscum contagiosum lesions to the mount.

The common wart and normal skin preparations revealed no uniform particles, but merely



FIG. 3.

Papilloma yielding virus-like particles as shown in Fig. 1. Base is at bottom. Hematoxylin-eosin. Magnification  $\times 30$ .

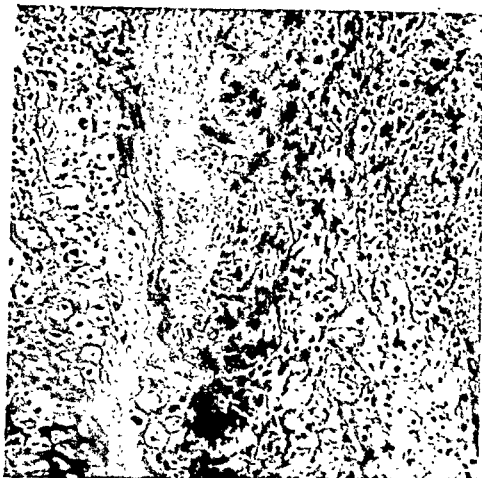


FIG. 4.

Higher power of a portion of Fig. 3—showing the prominent cytoplasmic bodies and looseness of lower cornified layers. Hematoxylin-eosin. Magnification  $\times 65$ .

amorphous scattered clumps of matter, collagen fibers, and spherical particles of varying diameter.

The histological appearance of the 5 papillomas yielding virus-like particles was similar (Fig. 3, 4, 5, 6). They showed irregular up-growths of thickened epidermis overlying elongated papillae containing fine blood ves-

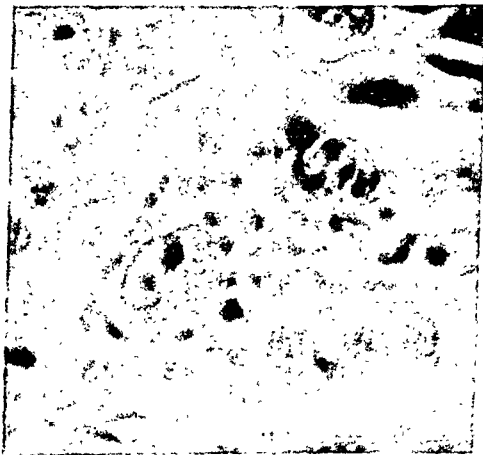


FIG. 5.

Same as Fig. 3. Note the single round intranuclear inclusion bodies photographing less black than the adjacent irregular chromatin masses. Solid cytoplasmic bodies are evident. Hematoxylin-eosin. Magnification  $\times 1200$ .

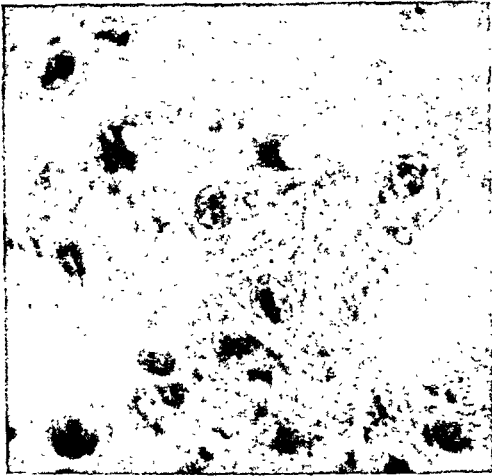


FIG. 6.

Same as Fig. 3. Both solid and vesicular cytoplasmic masses are conspicuous. Intranuclear inclusion bodies are also present. Hematoxylin-eosin. Magnification  $\times 600$ .



FIG. 7.

Common wart. Base is at bottom. Hematoxylin-eosin. Magnification  $\times 30$ .

sels and delicate connective tissue. There was a great increase in the thickness of the stratum corneum with persistence of pyknotic nuclei in the lower layers where the keratinized substance was loosely arranged and not as compact as at the surface. Significantly, intranuclear inclusion bodies were present in a large proportion of the cells of the papillomas, but not of the adjacent epidermis. These were first seen in the layer directly over the basal cells, and were evident in all of the more superficial layers. The inclusion bodies were round, stained with eosin and not hematoxylin; they were Feulgen negative and demonstrated little or no basophilia with methylene blue. Granules were present in the cytoplasm of the lowest row of cells that contained inclusion bodies. These granules when small were solid, but acquired a vacuole as they increased in size finally becoming oval or pointed in shape. Both the granules and the vesicular bodies stained with hematoxylin but had almost no affinity for the basic dye methylene blue and were Feulgen negative. At the level of the stratum granulosum they apparently became transformed into large solidly staining cytoplasmic bodies that persisted into the stratum corneum. Their affinity for hematoxylin was gradually replaced by a

strong eosinophilia by the time they had reached the stratum corneum in the region showing the parakeratosis. No elementary bodies were seen in any of the stages in Giemsa preparations.

The lesions of *molluscum contagiosum* were classical: a localized zone of hyperplasia of the epidermis with eosinophilic bodies filling the cytoplasm, displacing the nuclei of the cells of the upper Malpighian layers and persisting into the stratum corneum where they eventually became hyaline and fragmented. The molluscum bodies were Feulgen positive. The common warts had the usual histological appearance (Fig. 7): papillomas composed of hyperplastic epidermis covering prolonged papillary processes bearing vessels and a delicate stroma. Considerable hyperkeratosis with variable parakeratosis was present.

Search through the accumulated material of the laboratory of surgical pathology of the New Haven Hospital revealed 31 other skin lesions histologically similar to that of the inclusion body papillomas described above. The ages of these patients ranged from  $2\frac{1}{2}$  to 68 years. There were also 158 typical verrucae from individuals 5 to 80 years of age.

*Summary.* Virus-like particles that tend to be arranged in a crystalline-like pattern have been observed by electron microscopy in a

TABLE I.

Clinical diagnosis	No.	Electron microscopy			Pathology	
		Spherical particles	Spherical particles in crystalline-like array	Brick-shaped bodies	Intranuclear inclusion bodies	Molluscum bodies
Intranuclear inclusion body papilloma	5	5	4	—	5	—
Molluscum contagiosum	2	—	—	2	—	2
Common warts	9	—	—	—	—	—
Normal skin	1	—	—	—	—	—

study of suspensions of 5 skin papillomas. Histological examination of the same tissues revealed intranuclear inclusion bodies and characteristic cytoplasmic masses in the cells of the hyperplastic epidermis. Thirty-one

lesions with similar histological appearance were encountered along with 158 typical verrucae in a review of surgical pathological material.

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### Studies on the Thromboplastic Agent in Plasma. (17329)

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Thromboplastic preparations can conveniently be divided into two categories, those containing proteins and those which are lipids free from protein. In a recent study on the mode of action of Dicumarol<sup>1</sup> evidence was presented indicating that the thromboplastic agent in plasma responsible for the clotting of recalcified plasma had the physiological characteristics of a lipid thromboplastic agent. Further evidence has been obtained consistent with this view and will be presented in this paper. This evidence depends on a demonstration of a direct proportionality between the coagulation time obtained with the thromboplastic lipid and the coagulation time of a control sample of the same plasma which was recalcified and to which no thromboplastic agent was added. Such a relationship can be explained most simply on the basis that the thromboplastic agent in plasma has the characteristics of the lipid thromboplastic agent.

Such a relationship also implies, as will be discussed later, a mutual dependence on some limiting factor found in plasma.

*Methods.* The thromboplastic lipid was prepared from beef brain as outlined in the previous study.<sup>2</sup> It was stabilized by adding hydroquinone to make up 1% of its weight. This substance is an antioxidant that prevents the autoxidation of the preparation and its subsequent loss of activity.<sup>3</sup> In assaying the thromboplastic lipid a 0.2% emulsion in 0.9% NaCl was prepared from the dry material. The coagulation times of recalcified dog plasma were determined at room temperature with and without the thromboplastic lipid on 22 different occasions extending over a four month period. The plasma was obtained by withdrawing 9 parts of blood from the femoral artery of an unanaesthetized dog

<sup>2</sup> Hays, H. W., and Lein, J., *Arch. Biochem.*, 1945, 7, 69.

<sup>3</sup> Lein, J., and Lein, P. S., *Proc. Soc. Exp. Biol. and Med.*, 1949, 70, 446

<sup>1</sup> Lein, J., and Lein, P. S., *Am. J. Physiol.*, 1948, 155, 394.

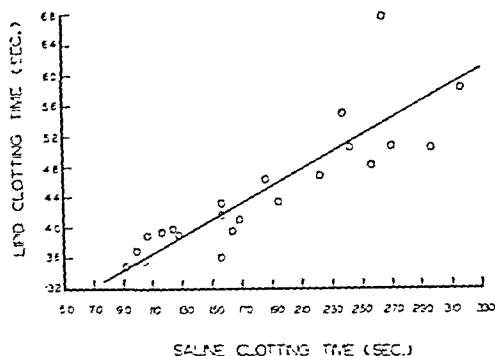


FIG. 1.

Linear relationship between clotting times obtained with and without the addition of the thromboplastic lipid to various samples of plasma.

directly into 1 part of 0.13 M sodium citrate. The blood was centrifuged for 30 minutes at 2,500 r.p.m. and the upper three-fourths of the plasma used. The clotting mixture consisted of 0.6 cc plasma, 0.2 cc of 1%  $\text{CaCl}_2$ , and 0.2 cc of either 0.9% NaCl or of 0.2% thromboplastic lipid emulsion in 0.9% NaCl. The  $\text{CaCl}_2$  solution was always added last and the coagulation time determined with a stop watch from the time of mixing of the clotting mixture to the time the tube could be inverted with no flow of its contents. The tubes used were small test tubes 10 x 75 mm. Two determinations were done in each case and the average used providing the average deviation did not exceed 10% of the mean. In a few cases where the deviations were greater, additional determinations were carried out.

**Results.** There was a considerable variation in the coagulation times of different samples of plasma. This was true in those determinations in which the thromboplastic agent was added as well as those in which only saline was added. The variation, however, was not random. The results indicate a definite relationship between the clotting time obtained without the addition of the thromboplastic lipid and that obtained with its addition. The results are presented graphically in Fig. 1, each of the points representing determinations on a different sample of plasma. The points were fitted by a straight line using the method of least squares. The significance of the regression coefficient was tested statistically by the null hypothesis<sup>4</sup> and was found to be

highly significant ( $df = 20$ ,  $t = 12.3$ ).

**Discussion.** The shortening of coagulation time by the addition of thromboplastic agents coupled with the fact that there is a great excess of prothrombin<sup>5</sup> and of fibrinogen<sup>6</sup> in normal plasma makes it evident that a limiting factor in coagulation when calcium ions are added in optimal quantities is the amount of thromboplastic agent present. The results have indicated that a linear relationship exists between the accelerated clotting time caused by the addition of the thromboplastic lipid and the clotting time of the plasma without this addition. The possibility that this dependence is due merely to the additive effect of 2 thromboplastic substances is excluded since the concentration of the lipid used was in excess of the amount necessary to produce the maximum shortening of coagulation time.<sup>2</sup> With this possibility ruled out, it becomes difficult to explain the results in terms of the classical ideas of thromboplastin action. If the lipid thromboplastic agent reacts with prothrombin in the presence of calcium ions to form thrombin, one would not expect there to be such a dependence as was demonstrated.

The results are believed to be explained most simply by the hypothesis that both the thromboplastic agent in the plasma and the thromboplastic lipid have the same mechanism of action and that the clotting of plasma by both of these agents depends on some limiting factor found in the plasma. The latter assumption is necessary since the results are obtained with optimal quantities of the thromboplastic lipid. The variation of clotting times of the different plasma samples is thought to be due to the variation in the plasma factor since prothrombin is present in more than adequate concentrations. According to this view the lipid thromboplastic agent prepared from tissues requires another factor found in plasma in order to become active. This is not true for protein thromboplastic agents since the work of Quick<sup>7</sup> and

<sup>4</sup> Snedecor, G. W., *Statistical Methods*, Iowa State College Press, Ames, 3rd ed., 118.

<sup>5</sup> Tanturi, C. A., and Banfi, R. F., *J. Lab. and Clin. Med.*, 1946, **31**, 703.

<sup>6</sup> Witts, L. J., *J. Path. and Bact.*, 1942, **54**, 516.

<sup>7</sup> Quick, A. J., *Science*, 1940, **92**, 113.



others showed that the plasma clotting times with these agents are absolute and depend only on the prothrombin concentration when it becomes limiting.

*Summary.* Statistical evidence is presented for a relationship between the coagulation time of recalcified plasma and the coagulation time accelerated by the addition of a throm-

boplastic lipid. This relationship is thought to imply that the thromboplastic substance in plasma responsible for the clotting of recalcified plasma has the same physiological characteristics as the lipid thromboplastic agent and that both require for activity some limiting factor present in plasma.

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### Effect on Prothrombin of Acute Massive Plasmapheresis with Simultaneous Chloroform Intoxication. (17330)

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From the earliest work on blood clotting, plasma prothrombin has been recognized as an integral component in the mechanism of thrombin formation. The effects on prothrombin of diverse pathologic and physiologic states have been studied extensively in the clinic and laboratory. Because prothrombin is so intimately related to liver function, an appreciable understanding of the origin, mode of action, and utilization of prothrombin may be acquired from subjecting the liver to various abnormal chemical and physical conditions. Experimental evidence and clinical experience with certain forms of liver and biliary tract disease indicate that plasma prothrombin is produced through the vital activity of the liver.<sup>1-7</sup> Thus experimentally induced hepatic necrosis,<sup>1,2</sup> and partial<sup>3</sup> or complete hepatectomy<sup>4,5</sup> result in hypoprothrombinemia.

Many factors, in addition to the fundamental integrity of the liver, are known to operate

in the maintenance of plasma prothrombin. An adequate intake of Vitamin K is essential. Certain drugs, notably dicumarol, cause hypoprothrombinemia without any morphologic evidence of damage to liver tissue. Yet very little is known about the mechanism by which prothrombin is produced and utilized in the body.

There is considerable evidence to suggest that plasma prothrombin will gradually disappear over a period of 1-3 days if the production of new prothrombin is impaired. Thus the minimum prothrombin concentration is reached in 36-48 hours following severe chloroform poisoning,<sup>1,2</sup> or partial hepatectomy.<sup>5</sup> If animals are rendered hypoprothrombinemic as a result of Vitamin K deficiency,<sup>8</sup> or dicumarol poisoning,<sup>9</sup> transfusions with normal blood result in a temporary elevation of prothrombin concentration. The effect of transfusions lasts only 1-2 days. Purified bovine prothrombin administered intravenously lasts 2-3 days in a dog made hypoprothrombinemic with dicumarol.<sup>10</sup> On the other hand, when prothrombin production is impaired, the tendency of plasma prothrombin concentration

<sup>1</sup> Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

<sup>2</sup> Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, 1937, **66**, 801.

<sup>3</sup> Warner, E. D., *J. Exp. Med.*, 1938, **68**, 831.

<sup>4</sup> Warren, R., and Rhoades, J. E., *Am. J. M. Sc.*, 1939, **198**, 193.

<sup>5</sup> Andrus, W. D., Lord, J. W., and Moore, R. A., *Surgery*, 1939, **6**, 899.

<sup>6</sup> Bollman, J. L., Butt, H. R., and Snell, A. M., *J.A.M.A.*, 1940, **115**, 1087.

<sup>7</sup> Brinkhous, K. M., *Medicine*, 1940, **19**, 329.

<sup>8</sup> Smith, H. P., Warner, E. D., Brinkhous, K. M., and Seegers, W. H., *J. Exp. Med.*, 1938, **67**, 911.

<sup>9</sup> Quick, A. J., *The Hemorrhagic Diseases*, Charles C. Thomas, 1942, p. 283.

<sup>10</sup> McGinty, D. A., Seegers, W. H., Pfeiffer, C. C., and Loew, E. R., *Science*, 1942, **90**, 540.

to stabilize at a subnormal level, rather than to continue to decrease, suggests a decrease in the rate of prothrombin utilization. Thus, with some types of liver disease, with continuous dicumarol administration, or with a continuous but inadequate Vitamin K intake, the prothrombin concentration tends to reach an equilibrium. Were the utilization rate constant, it would seem that the decline resulting from inadequate production should continue. Also, the rapid fall of prothrombin concentration following total hepatectomy<sup>4,5</sup> suggests that prothrombin "turn over" may be more rapid than indicated by experiments which impair production.

The purpose of the experiments reported in this paper was to determine the effect of massive plasmapheresis on the disappearance rate of prothrombin from the blood stream after severe chloroform intoxication, and to correlate the changes in prothrombin concentration with those of the total protein content of the plasma in dogs.

The data resulting from the present experiments entirely confirm previous findings<sup>1-7</sup> relative to the essential role of the liver in the manufacture of prothrombin. In addition, the experiments indicate that 1) no significant reserve stores of prothrombin exist, 2) when the prothrombin production rate is decreased, there is a concomitant decrease in the rate of its utilization, and 3) following chloroform administration, the impairment of the prothrombin producing capacity of the liver develops gradually over a period of 1-2 days.

**Methods.** Adult mongrel dogs, weighing from 5.3-12.9 kg, were used in all experiments. No attempt was made to control the diets rigidly, nor was any correlation between the diet and experimental observations noted. The low protein, high carbohydrate diet consisted of hospital scraps which contained bread, primarily, with very little meat. The stock diet was composed of hospital scraps supplemented with purina dog chow. The high protein diet consisted of meat and dog chow only. Donor blood was obtained from dogs maintained on a stock diet. Following removal of the plasma by centrifugation, the cells were washed twice with, and subsequently resuspended in, Locke's solution. No col-

TABLE I. Analysis on Data on Dogs Subjected to Plasmapheresis and/or Chloroform Intoxication.

Dog	45-1	45-2	45-3	45-4a	45-2	45-4b	45-8	45-9	45-10a	45-4c	45-10b	45-14	45-9	45-16
Type of experiment	P&C*	P&C	C	C	P	P&C	P&C	P&C	P&C	P	P	P	P&C	P&C
Hours of fasting before CHCl <sub>3</sub> and/or pheresis	0	30	50	50	0	50	72	50	50	50	0	50	50	50
% calculated blood volume removed by pheresis	102	65	—	—	69	73	84	95	95	87	87	78	105	69
% plasma proteins removed by pheresis	50	36	—	—	35	51	34	55	54	37	35	45	—	—
% prothrombin removed by pheresis	69	52	—	—	51	55	48	78	94	73	78	71	86	64
Condition of dog after pheresis and/or CHCl <sub>3</sub>	Death 2½ hr	Shock	Good	Good	Good	Shock	Shock	Shock	Shock	Shock	Good	Good	Shock	Good
Complete recovery of prothrombin (days)	—	18	8	8	1	10	8	6	6	2	2	2	7	6

\* P = Plasmapheresis, C = Chloroform.

loid substances were supplemented. A period of starvation preceded the chloroform anesthesia. (Table I.) Anesthesia was induced first with ether. The dogs subjected to chloroform and plasmapheresis were given deep chloroform anesthesia for from 75-100 minutes. Under ether anesthesia, immediately following chloroform, the femoral vessels were exposed and cannulated. The bottle containing warmed donor blood cell suspension was attached to an air pressure system so that the transfused blood entered the vein at virtually the same rate as blood was removed from the artery. Throughout the actual exchange, equal volumes of recipient and donor blood were constantly maintained. The rate of exchange was not allowed to exceed 100 cc per minute. The fraction of the calculated blood volume removed is indicated in per cent in Table I. The calculated blood volume was based on 90 cc per kg of body weight. Naturally, the blood removed during the pheresis procedure represents an admixture of the recipient's own blood and donor cells. The percentage of prothrombin and plasma proteins removed by the pheresis procedure was determined by calculation of differences of the levels before and immediately after plasmapheresis. Blood for the analyses was drawn from the jugular vein into a syringe wet with oxalate solution, and gently expressed into an 8 cc hematocrit tube containing 1.85% potassium oxalate. Prothrombin determinations were done by the two-stage method of Warner and Smith.<sup>1,2</sup> Crude lung extract which is rather rich in accelerator factor(s) was used as the source of thromboplastin. Protein values were determined by the semi-micro Kjeldahl method using the Parnas-Wagner distillation apparatus.

**Experimental Observations.** Prothrombin, total plasma protein, and hematocrit values in a typical experimental dog (45-4a) following prolonged chloroform anesthesia are recorded graphically in Fig. 1. The fall of prothrombin is extreme, the lowest level occurring at 40-48 hours. It is at this time that the icteric index is maximal, although the first appearance of icteric plasma is usually noted several hours earlier. Depending upon the depth and length of chloroform anesthesia, and the de-

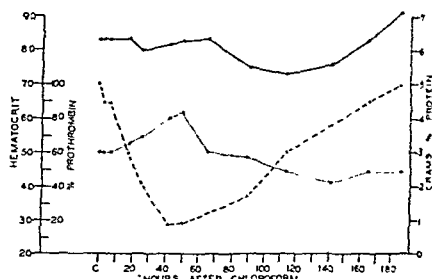


FIG. 1.

Effect of chloroform on prothrombin, plasma protein, and hematocrit values.

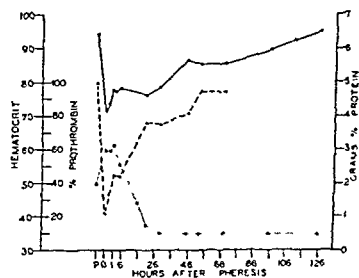


FIG. 2.

Effect of plasmapheresis on prothrombin, plasma protein, and hematocrit values.

gree of relative anoxia, the prothrombin concentration at the end of 48 hours may be very low. There is a concomitant elevation of hematocrit values indicating moderate hemoconcentration. Prothrombin recovery begins promptly on the third day and is complete by the eighth day. This recovery coincides with liver repair which begins in about 48 hours and is almost complete in 5-6 days. It is noteworthy that the plasma protein values fall moderately during the period of maximal liver regeneration and prothrombin recovery. This phenomenon was repeatedly observed, and its possible significance will be discussed later.

Fig. 2 shows the prothrombin, total plasma protein, and hematocrit values of a typical experimental dog (45-10b) following acute massive plasmapheresis. The precipitous fall of prothrombin and plasma proteins is roughly proportional to the amount of blood removed. At such low prothrombin levels, bleeding from venapuncture wounds frequently occurred; no spontaneous bleeding was observed, however. The recovery curve of prothrombin follows a rather characteristic

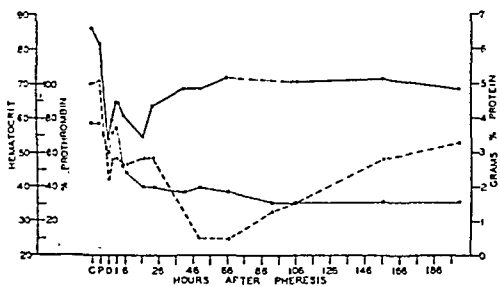


FIG. 3.

Effect of chloroform and plasmapheresis on prothrombin, plasma protein, and hematocrit values.

pattern, with a return to about 75% of normal in from 16 to 20 hours following plasmapheresis. By the end of 50 hours, prothrombin values have returned to normal. The recovery rate of the total proteins is more prolonged. It will be noted that after an abrupt rise of approximately 20% above the minimum value, the plasma proteins remain relatively constant for a period of about 26 hours, while during the same period, the prothrombin recovery rate is maximal. From 26-46 hours, the recovery rates of both prothrombin and plasma proteins are virtually the same, although for the latter, this period marks the maximal recovery rate. At the end of 116 hours, the plasma protein level has returned to normal. The sharp rise followed by the abrupt fall of the hematocrit is adequately explained by hemoconcentration followed by relative anemia.

When dogs are subjected to both acute massive plasmapheresis and chloroform intoxication, the graphical result is similar in several respects to a superimposition of the chloroform curve upon the pheresis curve. There are certain significant differences, however. Fig. 3 shows the results of a typical experiment of this type (dog 45-4b). The fall of prothrombin concentration is abrupt following plasmapheresis. This is followed by an exceedingly rapid increase in prothrombin values, probably due to hemoconcentration initially. The prothrombin concentration is maintained at this elevated level for a period of approximately 20 hours. This is in sharp contrast to the very transitory elevation of plasma protein and hematocrit values. Nu-

merous attempts were made to alter the character of this segment of the prothrombin curve, but regardless of diet, the quantity of exchanged blood, or the degree of chloroform intoxication, a postpheresis plateau invariably was obtained. At about 26 hours, prothrombin values again begin to fall and continue to do so for an additional 20 hours. If one graphically compares the decrease in prothrombin concentration after 26 hours with that of the comparable period in dogs subjected to chloroform intoxication only, it is observed that the curves are similar. This indicates that the termination of the postpheresis plateau and the subsequent decline in prothrombin concentration is the result of liver damage initiated some 30 hours previously by chloroform. As in the case of chloroform alone, the minimum prothrombin concentration is reached by the end of 48 hours. This is followed by a slow, but progressive recovery period.

The first segment of the plasma protein curve (Fig. 3) follows the pattern of the prothrombin curve rather closely. The rapid fall of plasma proteins parallels the fall of prothrombin. In every instance, the edema level was reached or exceeded, but, probably because this was only transitory, no clinical edema was noted. The postpheresis recovery curves of plasma protein values were quite variable, but generally speaking, there was a transitory rise with a secondary fall comparable to that of the hematocrit values. In the experiment shown in Fig. 3, concomitant with the fall of prothrombin, incident to the hepatotoxic effect of chloroform, plasma protein values gradually rose from 3.5 g% to 5.2 g%. This level was maintained for approximately 80 hours, during which time prothrombin recovery was most rapid. The general pattern of the plasma protein curve showed considerable variation in different animals, however. In some animals, there was a definite but minimal fall of plasma proteins during the period of maximal liver injury. Often, an additional drop in plasma proteins was observed during maximal prothrombin recovery. In other animals, plasma protein recovery paralleled prothrombin recovery. The

hematocrit values frequently showed a slight decrease immediately following plasmapheresis. A transitory rise, presumably due to hemoconcentration, then occurred in all of the dogs. This rise lasted only one to 2 hours in contrast to the postpheresis rise in prothrombin values, however. Considerable hemolysis was unavoidable in the massive pheresis procedure so that even though the hematocrit values of donor cell suspension and recipient blood were virtually the same, many of the washed red blood cells were hemolyzed. The result was a moderate anemia. The hematocrit values leveled off at from 30 to 40% and gradually returned to normal after 2-3 weeks.

*Discussion.* The profound fall in plasma prothrombin concentration in dogs having been subjected to chloroform intoxication confirms the previous work of Warner, Smith, and Brinkhous.<sup>1,2</sup> The evidence indicates that the liver is the source of prothrombin production, since the fall of prothrombin concentration rather closely parallels the degree of liver injury, and the prothrombin returns to normal as the liver regenerates.

Kerr, Hurwitz, and Whipple,<sup>11</sup> showed that severe liver injury in dogs by means of phosphorous or chloroform intoxication will cause, or at least be associated with, a moderate fall in blood plasma proteins. With 1½ hours of chloroform anesthesia, the fall began on the second day and progressed for the next 3 or 4 days during which period liver repair was most active. With comparable liver injury, our data (Fig. 1) revealed a similar plasma protein response, the minimal level occurring at about 4½ days. For almost 3 days following the administration of chloroform, during which period the prothrombin concentration had reached its lowest level and had begun to return toward normal values, the plasma proteins had remained relatively stable at a normal level. This lag period, occurring concomitantly with that of the greatest liver injury, could be explained by the presence of reserve stores of plasma proteins, if one assumes that the

liver is the source of albumin and globulin and that the rate of utilization remains constant at a normal level. If this assumption is valid, there must be some decrease, if not a total arrest, in the rate of plasma protein production resulting from liver injury. The lag period might also be taken as evidence, although inconclusive, that tissues other than liver fabricate albumin and globulin. Nevertheless, the extensive work of Whipple, Madden *et al.* strongly favors the liver as the source of the plasma proteins.<sup>12</sup> There are at least two other possible explanations for the decline of plasma proteins during the period of liver repair and maximal prothrombin regeneration. A smooth line curve of the plotted data accentuates the fact that the decline of plasma proteins coincides with an increase in prothrombin concentration, thereby suggesting a relationship between the two. Although prothrombin is only a very small fraction of the total proteins, it is conceivable that in order for prothrombin to be fabricated rapidly following liver injury, relatively large amounts of plasma proteins are diverted from the circulation to provide a pool from which the prothrombin molecule is constructed. Or, it is possible that the circulating proteins are called upon to supply the amino acid moiety necessary to fabricate liver protein itself, since the fall in plasma proteins occurs during the period of regeneration of liver tissue.

The rapid removal of as much as 50% of the circulating prothrombin results in the expected abrupt reduction of the level in the blood. On the basis of evidence cited above, that prothrombin in the blood lasts 1-3 days,<sup>1,5,8-10</sup> the removal of such amounts of prothrombin in animals subjected to chloroform intoxication would be expected to result in a conspicuously lower prothrombin level throughout the remainder of the experimental period. On the contrary, plasmapheresis does not appear to increase the severity of the hypoprothrombinemia, nor does it appear to alter the rate of prothrombin regeneration during the recovery period. The postpheresis plateau in chloroformed dogs could be explained on

<sup>11</sup> Kerr, W. J., Hurwitz, S. H., and Whipple, G. H., *Am. J. Physiol.*, 1918, **47**, 379.

<sup>12</sup> Madden, S. C., and Whipple, G. H., *Physiol. Rev.*, 1940, **20**, 194.

the basis of available prothrombin from reserve stores. Were this the case, however, once the reserves were exhausted, the prothrombin concentration would be expected to fall rapidly to a point below that of the chloroformed animals not subjected to plasmapheresis. Also, the rate of disappearance of prothrombin following total hepatectomy suggests that no significant stores exist. Further, with massive plasmapheresis in the normal animal, the fall in prothrombin concentration approximates the expected decrease from the amount of blood exchanged, and the rate of recovery is comparable to that seen in chronic Vitamin K deficiency following administration of specific therapy. Were appreciable quantities of prothrombin present in stores, it would be expected that the fall would be less and the recovery rate more rapid in the acute pheresis animal.

The relative ineffectiveness of massive plasmapheresis to materially alter the prothrombin level beyond 24-36 hours, despite greatly impaired liver function, could be explained on the basis of a very rapid turnover of the plasma prothrombin. Thus, if under normal conditions, the prothrombin of the blood was replaced several times per hour, the amount removed by plasmapheresis would become relatively insignificant. However, such rapidity of turnover does not seem compatible with the rate of fall of prothrombin following total hepatectomy.<sup>4,5</sup>

Evidence now available suggests that for any given level of hepatic function relative to prothrombin production, a balance is established between the rate of utilization and rate of formation. As a result, the plasma prothrombin becomes stabilized at a subnormal level, the height of which is determined more by productive capacity, than by any semi-constant rate of utilization. This is in accord with the tendency for the plasma prothrombin to become stabilized at a subnormal level when animals are maintained on a low Vitamin K or continuous dicumarol intake.

Our observations would strongly indicate, therefore, that no significant reserve stores of prothrombin exist, but rather that a nice balance between prothrombin production and utilization best explains the experimental find-

ings. Indeed, even the postpheresis plateau is probably an artefact, since if we compare the fluctuation of plasma protein and prothrombin values with those of the hematocrit, it will be seen that fluctuations of plasma volume might well result in an apparent increase per unit volume of plasma proteins and prothrombin.

Calculations involving total grams of plasma proteins and total units of prothrombin, respectively, 1) before plasmapheresis, 2) actually removed, and 3) remaining in the circulation, 1-10 minutes after pheresis, were made. The respective values were corrected for oxalate and hematocrit. The results were in accord with the fact that reserve stores of plasma protein exist, but indicated that no significant, if any, reserve stores of prothrombin are present. Since no total plasma volume determinations were performed, however, the calculations are inconclusive. Wide fluctuations in total plasma volume could readily distort the total values of prothrombin and plasma proteins.

From the experimental data, it can also be concluded that hepatotoxic damage with the concomitant decrease in prothrombin concentration incident to chloroform intoxication, is a progressive phenomenon which develops gradually over a period of 1-2 days. If the liver were completely functionless after the first hour of chloroform, the postpheresis plateau would then be impossible. Rather the prothrombin would continue to fall at a rate determined by utilization alone.

*Summary.* Dogs subjected to both chloroform intoxication and acute massive plasmapheresis reveal changes in prothrombin concentration which rather conclusively indicate that no significant reserve stores of prothrombin exist. The stabilization of prothrombin at subnormal levels, as well as the rate of recovery of prothrombin, represents, instead, a balanced equilibrium between production and utilization. The data also substantiate the impression that hepatotoxic injury, with a concomitant decrease in prothrombin concentration, is a gradual progressive phenomenon.

# Electrocardiographic Changes Produced on the Syrian Hamster (*Cricetus auratus*) by Diphtherial Toxins. (17331)

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The golden hamster, *Cricetus auratus*, is sensitive to diphtherial toxin and exhibits neurotoxic symptoms when toxins exposed to alkaline reactions are administered, whereas toxins kept at slightly acid or neutral reactions do not exert apparent effects on its nervous system.<sup>1,2</sup> Since the clinical syndrome of diphtheria is characterized by neurological disturbances as well as abnormalities in the cardiac function, it was decided to study the effect of diphtherial toxins on the heart of the golden hamster.

**Methods.** Toxin samples were prepared from filtrates of 8 day cultures of *Corynebacterium diphtheriae* (Park No. 8 strain). With the aid of highly diluted HCl or NaOH the toxins were brought to the proper pH level and kept at it for 8 days at 30°C as described elsewhere.<sup>1,2</sup> Clinical and electrocardiographic examinations were made on a total of 24 hamsters; special attention was paid to the appearance of peripheral paralysis. Limb leads electrocardiograms were taken on a portable amplifier instrument. Small (0.5 : 1.2 cm) electrodes of German silver were applied after the skin had been rubbed with electrode jelly.

**Results.** In 5 apparently normal hamsters electrocardiograms were taken at 3 day intervals for one month. They showed within this period a constant pattern, the various deflections being similar to those of the human electrocardiogram. The P wave was indistinct owing to the rapid heart rate, 300-400 per minute, as shown in Fig. 1, left side.

Ten hamsters received minimal lethal doses of a toxin kept at pH 6.7; their average survival time was 8 days. No neurotoxic symp-

toms appeared; daily electrocardiograms did not reveal any disturbance of the heart function. In 4 animals we succeeded in obtaining electrocardiograms 4 hours before death; they were found to be normal. At gross pathological examination the classical signs of diphtherial intoxication were found: edema and necrosis at the site of injection and hemorrhages in the adrenal glands.

Another group of 11 hamsters received minimal lethal doses of a toxin exposed to pH 7.3. In 5 of them paresis appeared and their electrocardiograms showed marked abnormalities, summarized in the upper part of Table I. These changes preceded by some days the appearance of neurological abnormalities. In 6 other animals the electrocardiogram and the neurological functions remained normal.

A third group of 8 animals received minimal lethal doses of a toxin exposed to pH 8.5; their average survival time was 21 days. All of them showed paresis and marked electrocardiographic disturbances, summarized in the second part of Table I. (cf. Fig. 1, right).

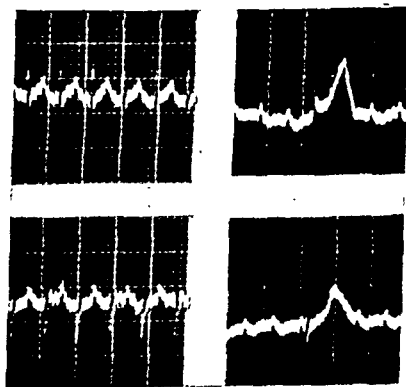


FIG. 1.

Left: Electrocardiogram (leads II and III) in a normal hamster.

Right: Auriculo-ventricular block after the injection of a diphtherial toxin kept at pH 8.5.

<sup>1</sup> Olitzki, L., Stuczynski, L. A., and Grossowicz, N., 4th Internat. Cong. f. Microbiol. Copenhagen, 1947; Rep. Proc., 1949, p. 180.

<sup>2</sup> Olitzki, L., Stuczynski, L. A., and Grossowicz, N., J. Immunol., 1948, 60, 419.

TABLE I.

Paresis and Heart Disturbances Produced in Hamsters by Minimal Lethal Doses of Diphtherial Toxins Exposed to Alkaline Reactions.

No. hamster	Toxin kept at pH	First appearance (days) of disturbances of the		Survival, days	Functional abnormalities observed
		Nervous system	Heart function		
1	7.3	4	3	5	Ectopic beats
2	7.3	8	6	9	" "
3	7.3	10	7	25	" "
4	7.3	5	4	6	Partial auriculo-ventricular block
5	7.3	11	7	12	Ectopic beats
6	8.5	23	23	30	" " , later total auriculo-ventricular block
7	8.5	25	25	28	Like No. 6
8	8.5	7	7	9	total auriculo-ventricular block
9	8.5	7	7	12	Like No. 8
10	8.5	22	22	28	Like No. 8
11	8.5	22	22	30	Ectopic beats
12	8.5	8	8	18	Partial auriculo-ventricular block
13	8.5	8	8	14	Like No. 12

TABLE II.

Paresis and Heart Disturbances Produced in Hamsters by Sublethal Doses of Diphtherial Toxins Exposed to Alkaline Reactions.

No. hamster	Toxin kept at pH	Paresis, day of		Ectopic beats, day of	
		First appearance	Recovery	First appearance	Recovery
1	7.3	16	46	9	90
2	8.5	10	44	20	150
3	9.0	17	35	22	150
4	9.0	17	45	22	270

In a group of 4 hamsters the action of sublethal doses (0.2-0.5 MLD) of toxins was examined. In this group the neurotoxic symptoms were of a transient character and disappeared about 3 to 4 weeks after their first appearance. The electrocardiographic abnormalities, on the other hand, persisted much longer and in one hamster were apparent 9 months after the injection of the toxin. (Table II).

*Summary and conclusions.* Diphtherial toxins exposed to alkaline reactions produced in the golden hamster, in addition to neurotoxic symptoms, marked disturbances in the heart function as indicated by electrocardiographic

abnormalities. Acid-treated toxins on the other hand, did not cause any electrocardiographic changes. The first appearance of the electrocardiographic changes frequently preceded that of the paralytic symptoms, and when sublethal doses were administered, the electrocardiogram remained abnormal even after the neurological symptoms had disappeared. Electrocardiographic examination of the heart function seems, therefore, to be a more sensitive method of measuring the action of certain diphtherial toxins than the observation of the neurotoxic symptoms.

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# Electrocardiographic Changes Produced on the Syrian Hamster (*Cricetus auratus*) by Diphtherial Toxins. (17331)

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The golden hamster, *Cricetus auratus*, is sensitive to diphtherial toxin and exhibits neurotoxic symptoms when toxins exposed to alkaline reactions are administered, whereas toxins kept at slightly acid or neutral reactions do not exert apparent effects on its nervous system.<sup>1,2</sup> Since the clinical syndrome of diphtheria is characterized by neurological disturbances as well as abnormalities in the cardiac function, it was decided to study the effect of diphtherial toxins on the heart of the golden hamster.

**Methods.** Toxin samples were prepared from filtrates of 8 day cultures of *Corynebacterium diphtheriae* (Park No. 8 strain). With the aid of highly diluted HCl or NaOH the toxins were brought to the proper pH level and kept at it for 8 days at 30°C as described elsewhere.<sup>1,2</sup> Clinical and electrocardiographic examinations were made on a total of 24 hamsters; special attention was paid to the appearance of peripheral paralysis. Limb leads electrocardiograms were taken on a portable amplifier instrument. Small (0.5 : 1.2 cm) electrodes of German silver were applied after the skin had been rubbed with electrode jelly.

**Results.** In 5 apparently normal hamsters electrocardiograms were taken at 3 day intervals for one month. They showed within this period a constant pattern, the various deflections being similar to those of the human electrocardiogram. The P wave was indistinct owing to the rapid heart rate, 300-400 per minute, as shown in Fig. 1, left side.

Ten hamsters received minimal lethal doses of a toxin kept at pH 6.7; their average survival time was 8 days. No neurotoxic symp-

toms appeared; daily electrocardiograms did not reveal any disturbance of the heart function. In 4 animals we succeeded in obtaining electrocardiograms 4 hours before death; they were found to be normal. At gross pathological examination the classical signs of diphtherial intoxication were found: edema and necrosis at the site of injection and hemorrhages in the adrenal glands.

Another group of 11 hamsters received minimal lethal doses of a toxin exposed to pH 7.3. In 5 of them paresis appeared and their electrocardiograms showed marked abnormalities, summarized in the upper part of Table I. These changes preceded by some days the appearance of neurological abnormalities. In 6 other animals the electrocardiogram and the neurological functions remained normal.

A third group of 8 animals received minimal lethal doses of a toxin exposed to pH 8.5; their average survival time was 21 days. All of them showed paresis and marked electrocardiographic disturbances, summarized in the second part of Table I. (cf. Fig. 1, right).

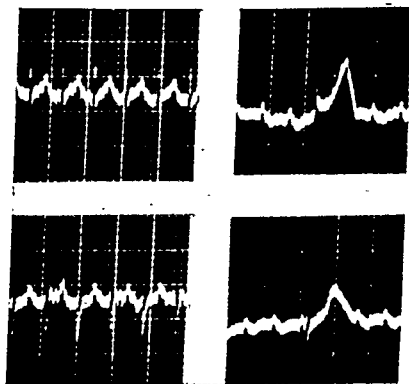


FIG. 1.

Left: Electrocardiogram (leads II and III) in a normal hamster.

Right: Auriculo-ventricular block after the injection of a diphtherial toxin kept at pH 8.5.

<sup>1</sup> Olitzki, L., Stuczynski, L. A., and Grossowicz, N., 4th Internat. Cong. f. Microbiol. Copenhagen, 1947; Rep. Proc., 1949, p. 180.

<sup>2</sup> Olitzki, L., Stuczynski, L. A., and Grossowicz, N., J. Immunol., 1948, 60, 419.

calcium beta naphthol in the alkaline phosphatase method of Manheimer and Seligman, p-chloranilidophosphonic acid in the phosphamidase method, and palmitoyl choline chloride in preference to myristyl choline chloride in the cholinesterase method.

**Results.** The patterns of the different enzyme activities were consistent in the brains examined. Alkaline phosphatase was demonstrated in an apparently great quantity in the pigeon brain (Fig. 1) by both the methods of Gomori, and Manheimer and Seligman. This observation is noteworthy because Gomori<sup>17</sup> and Bourne<sup>18</sup> state that alkaline phosphatase is not found in brain tissue. On the other hand we could not demonstrate in the pigeon the great quantities of acid phosphatase which are reported by these workers to be present in the brains of other animals. However, the faint patterns which we did obtain were consistent. Although the results of this procedure are difficult to interpret, we have included them in our comparative analysis of the different enzyme patterns.

From observations restricted to a few clearly defined structures, we wish to point out some striking differences in the enzyme content of a few areas in the brain keeping in mind the questions raised in one of the preceding paragraphs concerning the validity of the methods employed.

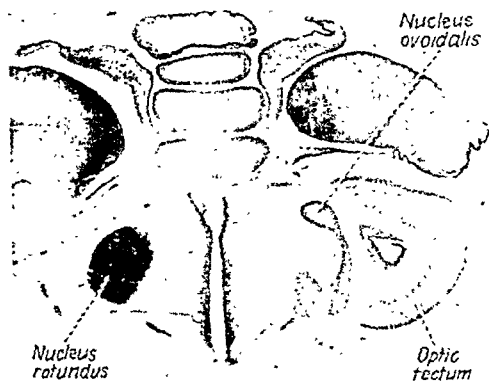


FIG. 2.

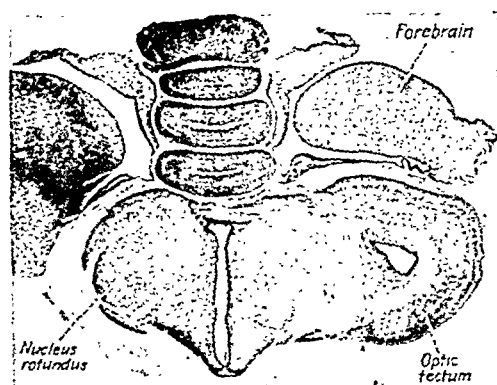


FIG. 3.

In adjacent sections the nucleus ovoidalis appears to be entirely lacking in alkaline phosphatase (Fig. 1), but seems very rich in cholinesterase (Fig. 2). Its outline may be traced in a preparation which demonstrates acid phosphatase, but it can hardly be distinguished from other structures in a phosphamidase preparation (Fig. 3) inasmuch as its content of phosphamidase is about the same as that of the surrounding brain tissue.

The nucleus rotundus is similarly conspicuous in cholinesterase preparations (Fig. 2) whereas its outline can be barely identified in acid phosphatase preparations. Its content of alkaline phosphatase is markedly less than that of surrounding structures (Fig. 1); in phosphamidase preparations its boundaries are indistinct (Fig. 3).

The occipital pole of the forebrain shows some acid phosphatase and a high content of phosphamidase (Fig. 3) and of cholinesterase

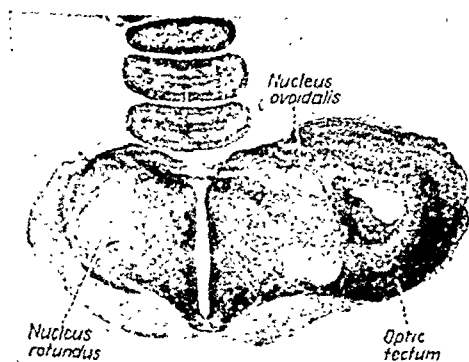


FIG. 1.

<sup>17</sup> Gomori, G., *J. Cell. and Comp. Physiol.*, 1941, 17, 71.

<sup>18</sup> Bourne, G., *Quart. J. Exp. Physiol.*, 1943, 32, 1.

# Distribution of Certain Enzymes in the Brain of the Pigeon.\* (17332)

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In the course of investigations dealing with the functional significance of cerebral capillary patterns, a relationship was found between the vascularity of certain neuropils and the number of mitochondria present.<sup>1</sup> The relationship was considered significant because mitochondria are carriers of enzymes.<sup>2-4</sup> This fact and the availability of histochemical methods for the demonstration of enzymes in tissues suggested a study of the distribution of enzymes in the brain as one aspect of the relationship between cerebral vascularization and brain metabolism.

**Materials and methods.**<sup>†</sup> Pigeon brains were used in this study because it was found from a survey of the brains of various vertebrates that the histochemical methods available for the demonstration of enzyme activities gave the best over all results in pigeon brains. The methods used were those of Gomori<sup>5</sup> and Manheimer and Seligman<sup>6</sup> for alkaline phosphatase, and those of Gomori for acid phosphatase,<sup>7</sup> phosphamidase,<sup>8</sup> and cholinesterase.<sup>9</sup> Although these methods represent valuable

additions to the existing histochemical methods, caution is required in the interpretation of the results that may be obtained from their application. Several investigators<sup>10-12</sup> have shown that there is a definite unavoidable destruction and loss of the phosphomonoesterases, particularly acid phosphatase, as a result of the fixing, embedding, and mounting processes to which the tissues are subjected. In fact, the validity of the assumption that the results obtained by the acid phosphatase method are wholly or at all due to the activity of this particular enzyme has been seriously questioned.<sup>11,13,14</sup> The accuracy of the localization of alkaline phosphatase activity has been doubted,<sup>15</sup> but the sharp boundaries in our pictures indicate that there occurred little if any diffusion. In regard to the demonstration of cholinesterase activity Koelle and Friedenwald<sup>16</sup> suggest that the method of Gomori localizes only a non-specific enzyme and that there may be other undemonstrated cholinesterases present which have different substrate specificities.

In preparation for the enzyme determinations the tissues were fixed in chilled acetone or absolute alcohol, were transferred to benzene and were infiltrated with and embedded in paraffin in a vacuum oven. The sections were cut at 5 $\mu$ . The substrates used were sodium alpha glycerophosphate in the acid and alkaline phosphatase methods of Gomori,

\* This paper reports research undertaken under contract with the Office of Naval Research. The conclusions contained in this report are those of the authors and do not necessarily reflect the views of the Office of Naval Research.

<sup>1</sup> Scharrer, E., *J. Comp. Neur.*, 1945, **83**, 237.

<sup>2</sup> Warburg, O., *Pflüger's Arch. f. d. ges. Physiol.*, 1913, **154**, 599.

<sup>3</sup> Lazarow, A., and Barron, E. S. G., *Anat. Rec.*, 1941, **79**, (suppl.), 41.

<sup>4</sup> Claude, A., A.A.A.S. Res. Confer. on Cancer, 1945, 223.

<sup>†</sup> We are greatly indebted to Dr. G. Gomori, University of Chicago, for helping us solve technical problems that arose in the course of the investigation.

<sup>5</sup> Gomori, G., *PROC. SOC. EXP. BIOL. AND MED.*, 1939, **42**, 23.

<sup>6</sup> Manheimer, L. H., and Seligman, A. M., *J. Nat. Cancer Inst.*, 1948, **9**, 181.

<sup>7</sup> Gomori, G., *Arch. Pathol.*, 1941, **32**, 189.

<sup>8</sup> Gomori, G., *PROC. SOC. EXP. BIOL. AND MED.*, 1948, **69**, 407.

<sup>9</sup> Gomori, G., *PROC. SOC. EXP. BIOL. AND MED.*, 1948, **63**, 354.

<sup>10</sup> Doyle, W. L., *PROC. SOC. EXP. BIOL. AND MED.*, 1948, **69**, 43.

<sup>11</sup> Smith, W. K., *Anat. Rec.*, 1948, **102**, 523.

<sup>12</sup> Stafford, R. O., and Atkinson, W. B., *Science*, 1947, **107**, 279.

<sup>13</sup> Lassek, A. M., *Stain Tech.*, 1947, **22**, 133.

<sup>14</sup> Bartelmez, G. W., and Bensley, S. H., *Science*, 1947, **106**, 639.

<sup>15</sup> Jacoby, F., and Martin, B. F., *Nature*, 1949, **163**, 875.

<sup>16</sup> Koelle, G. B., and Friedenwald, J. S., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 617.

## A Method for Measuring Removal of Bacteria from the Blood by the Various Organs of the Intact Animal.\*† (17333)

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The ability of the body to remove foreign matter from the circulation has interested investigators since the nineteenth century. Wysokowitch<sup>1</sup> in 1886 noted that the cells of the vascular endothelium rapidly cleared the blood of pathogenic and non-pathogenic organisms. Aschoff<sup>2</sup> considered these cells as a functioning unit which he called the reticulo-endothelial system. By perfusing isolated organs Manwaring<sup>3</sup> found that the canine liver and spleen removed organisms much more rapidly than other organs. Studies on the rapidity of removal and the acceleration of removal by immunity were carried out by Bull<sup>4</sup> and Wright.<sup>5</sup> Cannon<sup>6</sup> and Sullivan<sup>7</sup> showed that after single intravenous injections, large numbers of organisms were found in the liver and spleen with few in the brain, lungs, muscle and thyroid. Beeson<sup>8</sup> in his study of subacute bacterial endocarditis noted the sparsity of organisms in hepatic venous blood. The object of the present report is to describe a method of quantitative estimation

of the rate of removal of bacteria from the blood stream in the intact animal.

**Methods.** Healthy 7 to 23 kg dogs and 2.6 to 3.1 kg albino male rabbits were used. Light anesthesia was maintained throughout the experiment by the use of intravenous sodium pentobarbital.

The hemolytic *Micrococcus aureus* employed was of human origin, coagulase positive, and it fermented mannite. The encapsulated *Klebsiella pneumoniae* type B was obtained from Dr. R. J. Dubos. The inoculum used for infusion consisted of a suitably diluted saline suspension of bacteria washed from the surface of Bacto tryptose agar after 5 to 6 hours of incubation at 37°C.

A controlled bacteremia was maintained by a continuous infusion into the femoral vein, the inferior vena cava, or the right auricle of the dog of 1 to 5 × 10<sup>5</sup> hemolytic *Micrococcus aureus* organisms per minute by means of a number 6 or 8 ureteral catheter introduced into the veins. A similar infusion of approximately 1 × 10<sup>4</sup> *Klebsiella pneumoniae* type B organisms per minute was accomplished in the rabbit using the superior vena cava. A constant rate of inflow was maintained by the use of a standard intravenous drip set or by the use of a syringe activated by a motor-driven plunger with variable gears allowing control of the rate of movement of the plunger. An initial relatively large dose of organisms was given to each animal in order to attain rapidly the desired blood bacterial count which was maintained thereafter by the constant inflow of bacteria. After 5 to 30 minutes blood samples for culture were drawn from various sites as indicated in Tables I through III.

In the dog, femoral and jugular venous and femoral arterial blood samples were drawn at approximately 15 minute intervals through an arterial-type needle left *in situ*. Hepatic

\* This work was supported by the Anna H. Hanes Research Fund.

† The authors wish to express their appreciation to Dr. D. T. Smith of the Department of Bacteriology for making available the media and certain other supplies which were used.

<sup>1</sup> Wysokowitch, W., *Z. f. Hyg. u. Inf.*, 1886, 1, 3.

<sup>2</sup> Aschoff, L., *Ergebn. Inn. Med. Kinderheilk*, 1924, 26, 1.

<sup>3</sup> Manwaring, W. H., and Fretsch, William, *J. Immunol.*, 1923, 8, 83.

<sup>4</sup> Bull, C. A., *J. Exp. Med.*, 1916, 24, 7.

<sup>5</sup> Wright, H. D., *J. Path. and Bact.*, 1927, 30, 185.

<sup>6</sup> Cannon, Paul R., Sullivan, F. L., and Neckermann, E. F., *J. Exp. Med.*, 1932, 55, 121.

<sup>7</sup> Sullivan, F. L., Neckermann, E. F., and Cannon, Paul R., *J. Immunol.*, 1934, 26, 49.

<sup>8</sup> Beeson, P., Brannon, E. S., and Warren, J. V., *J. Exp. Med.*, 1945, 81, 9.

(Fig. 2); but it is entirely free of alkaline phosphatase (Fig. 1).

The observation of layers of enzymatic activity in the optic tectum (Figs. 1-3) is of particular interest in view of the corresponding arrangement of cells, fibers, blood vessels, and mitochondria in this part of the brain. A more detailed description of the distribution of enzymes in the optic tectum will be given in a separate paper.

*Discussion.* The statements "free of" or "rich in" as made in the description of our preparations should, of course, not be taken to mean absolute quantitative values, since there is, as has been pointed out above, an unavoidable loss of enzymatic activity that occurs in the process of fixing, embedding and mounting the tissues.

Consequently the results which we have obtained are only relative. For example the nucleus ovoidalis and the occipital pole of the forebrain appear in our preparations free of alkaline phosphatase. This may be only the result of the loss of the relatively small amounts of the enzyme which they contained in the living state. Other adjoining brain tissue may have had so much more alkaline phosphatase to begin with that even after having been subjected to the same technical procedures there is still enough of the enzyme left to give a marked reaction with the methods employed. The fact however remains that there are demonstrable differences in enzyme content.

The distribution of cholinesterase activity shown in our preparations is of interest in comparison with the results of Feldberg and Vogt.<sup>19</sup> These authors assayed very small amounts of tissue taken from 40 different regions of the central nervous system; they found pronounced differences in the content of the enzyme or enzyme system synthesizing acetylcholine in the different regions examined. The conclusion of Feldberg and Vogt that in the central nervous system cholinergic neurons alternate with noncholinergic

ones suggests the possibility that our pictures of the distribution of cholinesterase in the pigeon brain may reflect the actual situation rather than inadequacies of the method.

It is evident that at present no conclusion should be drawn from these observations as to the differences in actual metabolic functions of various parts of the brain. But it is clear that such areas as the nucleus rotundus and the nucleus ovoidalis differ not only in anatomical structure but also in some way biochemically from the surrounding brain tissue. For instance processes which require an enzyme to catalyze the hydrolysis of phosphomonoesters within the pH range 9.0 to 9.4 evidently occur to a much smaller extent in the nucleus rotundus and nucleus ovoidalis than in the adjoining optic tectum. On the other hand the large amounts of cholinesterase in the nucleus rotundus and nucleus ovoidalis indicate that they consist largely of cholinergic neurons and that they are surrounded by brain tissue containing mainly non-cholinergic neurons. Whatever these differences may mean they are so pronounced that these structures are more conspicuous in histochemical preparations than in sections stained with ordinary histological stains.

*Summary and conclusions.* Histochemical methods were used to demonstrate the presence of alkaline and acid phosphatase, phosphamidase, and cholinesterase in the brain of pigeons. It was found that there are marked differences in the enzyme contents of various structures in the brain. This observation corroborates the concept of biochemical differences between different parts or different functional and anatomical systems that has evolved from the work of a number of investigators.<sup>‡</sup> The use of histochemical methods makes possible the more detailed study of the "Chemoarchitectonics" of the vertebrate brain which will be of interest when correlated with the cyto-, myelo- and angioarchitectonics.

<sup>‡</sup> The relevant literature will be discussed in another paper.

<sup>19</sup> Feldberg, W., and Vogt, M., *J. Physiol.*, 1948, **107**, 372.

TABLE II. Blood Levels of Bacteria and Percent Removal by Various Organs in Normal Dogs.

Duration i.v. admin. bact. (min.)	Splenic vein						Pulmonary artery						Jugular vein					
	9			10			11			12			13					
	F.A.		%Es	F.A.		S.V.	F.A.		%Es	F.A.		P.A.	F.A.		P.A.	J.V.		F.A.
	S.V.																	
15																139	123	14
30									40	62						246	46	89.0
45	73			208			46	41	46	41	103	129	103			400	85	81.3
60	63			162		26	41	32	82.9		123	111	123			673	190	76.1
75							28	28										44.1
90	32			115		22	25	27	80.0		90	103				166,000	56,000	97.4
105	70	14	80.0	126		6	52	45			322	327				27,000	25,000	18' after
120	70	40	49.4				46	38	95.4		426	411						89.6
135							41	44			364	411						240
150							28	16										240
																		105

S.V.—Splenic venous.

P.A.—Pulmonary arterial.

J.V.—Jugular venous.

%Es—% splenic removal (F.A.—S.V.  $\times 100$ ).

F.A.

Other abbreviations as in preceding table.

venous, renal venous and pulmonary arterial blood samples were obtained through indwelling number 6 or 8 ureteral catheters introduced into the vessel via a jugular vein under fluoroscopic visualization. The technic of venous catheterization used in these experiments has been described by Courmand and Ranges.<sup>9</sup> In each instance the right lobe of the liver was catheterized. Splenic venous blood samples were obtained by repeated venipunctures or from an arterial-type needle left *in situ* under direct visualization after opening the abdomen.

In the rabbit mixed blood samples were obtained at approximately 7 minute intervals in initial preparations by repeated cardiac punctures and in subsequent preparations through an indwelling number 6 ureteral catheter introduced via a jugular vein into the inferior vena cava to the lower lumbar level. In each instance the left lobe of the liver was catheterized.

In numerous experiments the animals were sacrificed at the close of the observation period, and the position of the catheters was checked then by direct visualization.

Experiments revealed that the preliminary withdrawal of 4 ml of blood sufficed to clear the catheter of its contents so that the subsequent sample represented the blood present in the lumen of the catheterized vessel. Such clearance was done in every instance prior to the withdrawal of a blood sample for study.

Duplicate pour plates were made with blood samples of 0.5 to 1.0 ml volume in Bacto tryptose agar, serial saline dilutions of the blood being used when necessary to obtain countable plates.

**Results.** In Fig. 1 a representative experiment on a dog is charted. The bacterial counts in the femoral venous and femoral arterial blood varied somewhat during the course of each experiment. This is attributed to a slight variation in the rate of inflow of organisms and to the fact that the number of viable bacteria in the saline suspension which was administered decreased during the course of each experiment, as shown by a comparison of the

<sup>9</sup> Courmand, A., and Ranges, H. A., *Proc. Soc. Exp. Biol. and Med.*, 1941, 40, 462.

## REMOVAL OF BACTERIA FROM BLOOD

TABLE I.  
Blood Levels of Bacteria and Percent Splanchnic Removal in Normal Dogs.

Duration i.v. admin. baet. (min.)	1				2				3				4			
	F.V.	F.A.	H.V.	%Eh	F.V.	F.A.	H.V.	%Eh	F.V.	F.A.	H.V.	%Eh	F.V.	F.A.	H.V.	%Eh
30	284	352	153	56.5	515	457	98	78.7	453	559	82	85.3	421	335	42	87.4
45	356	377	27	92.8	460	467	96	79.3	477	477	165	65.4	364	329	60	81.7
60	345	362	66	81.8	345	446	120	73.6	444	444	147	66.9	184	290	28	90.3
75	357	376	76	78.7	336	427	94	77.7	484	484	79	83.0	199	437	27	93.8
90	292	343	58	91.5	229	224	43	80.8	342	342	82	76.0	368	233	17	96.4
105	309	352	75	78.7	402	372	77	79.4	404	404	25	93.8	249	468	28	94.2
120	238	247	60	75.7	278	389	104	73.3					405	480	20	95.5
135	214	251	73	70.9	243	282	72	74.5					353	446		
150	244	292	61	79.1												
165	104	284	60	78.7												

Duration i.v. admin. baet. (min.)	5				6				7				8			
	F.V.	F.A.	H.V.	%Eh	F.A.	H.V.	%Eh		F.A.	H.V.	%Eh		F.V.	F.A.	H.V.	%Eh
30					68	62	8.8		152	76	50.0		145	129	24	81.4
45					153	23	85.0		171	79	53.8		143	132	24	81.8
60	785	861	331	62.0	79	20	74.7		132	38	71.2					
75	826	903	426	53.0	113	45	60.2		129	56	56.6		92	80	18	44.4
90	1065	946	473	50.0									139	92	9	93.5
105																
120	841	973	372	62.0												
135	905	858	342	60.0												
150	729	788	278	65.0												

F.V.—Femoral venous.  
F.A.—Femoral arterial.

H.V.—Hepatic venous.

%Eh—% splanchnic removal (F.A.—H.V.  $\times$  100).  
F.A.

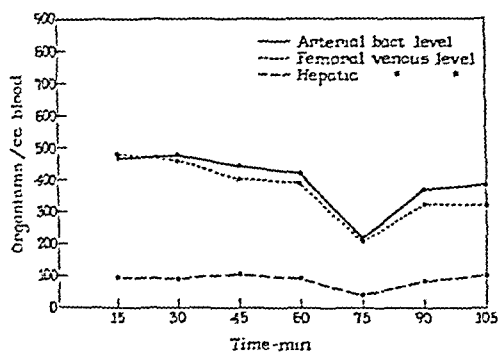


FIG. 1.

Chart showing bacteremia maintained over 105 minutes with arterial levels plotted against femoral and hepatic venous levels.

inoculum titres at the beginning and at the end of the observation period. The data permit comparison of counts on samples drawn simultaneously from different sites, however.

Table I records the counts of hemolytic *Micrococcus aureus* per ml of blood in a group of nine dogs, including one animal (No. 18) in which an arterial blood level of  $1 \times 10^5$  bacteria per ml was maintained throughout the experiment. The rate of removal of organism by the splanchnic circulation is recorded in percent calculated from the simultaneous hepatic venous and the femoral arterial counts. A high percentage of organisms was taken out by the splanchnic circulation, the average removal in 57 observations on nine dogs being  $74 \pm 16\%$  (S.D.). An analysis of femoral venous and femoral arterial bacterial counts based on 35 observations in 6 dogs showed that the removal of organisms in the peripheral circulation of the hind extremity was variable within wide limits, averaging  $12 \pm 14\%$  (S.D.).

Table II presents observations in a group of 5 dogs in which various other organs were studied for their ability to remove hemolytic *Micrococcus aureus* from the blood stream. In two dogs (Nos. 9 and 10) the bacterial counts per ml of blood from the femoral artery and the splenic vein were used to calculate the percent removal in the splenic circulation. The average removal by the spleen was  $78 \pm 17\%$  (S.D.) a rate which approximates closely that of the splanchnic circulation as a whole. In 2 animals (Nos.

11 and 12) no significant difference was found on comparison of the pulmonary arterial and the femoral arterial bacterial counts. In 6 observations on a single animal (No. 13), the jugular venous counts were  $24 \pm 27\%$  (S.D.) higher than the simultaneously determined femoral arterial counts. Preliminary studies in several dogs not included in the present series have indicated no consistent trend in the relationship of renal venous and superior mesenteric venous counts to femoral arterial counts.

Table III records the bacterial counts per ml of blood in a group of 7 rabbits receiving an encapsulated *Klebsiella pneumonia* Type B. The percent removal by the splanchnic circulation was calculated from the hepatic venous and the intracardiac or the inferior vena caval bacterial counts. Average splanchnic removal in 39 observations on 7 rabbits was  $20 \pm 10\%$  (S.D.), a degree of removal which is highly significant ( $p = < 0.01$ ).

**Discussion.** A constant intravenous bacterial infusion combined with the technic of venous catheterization provides a convenient method of determining the rate of removal of microorganisms by the organs draining directly into the caval system. The spleen must be studied by some method allowing puncture of the splenic vein. The method used here may be useful in studying the rate of removal of antigens and other particulate matter.

Cannon<sup>6</sup> studied the removal of the micrococcus from the blood stream by culturing weighed amounts of various organs. He showed the remarkable capacity of the animal to concentrate the injected organisms in the liver and spleen as well as the ability of these organs to destroy the micrococcus. The lungs did not concentrate the organisms after intravenous injection. Manwaring<sup>3</sup> found the extraction in the liver and spleen to be 80 and 60%, respectively. Beeson<sup>8</sup> found an average of 85% extraction in hepatic venous blood in human subacute bacterial endocarditis.

The figure for the total splanchnic removal of the hemolytic *Micrococcus aureus* in our dogs was  $74 \pm 16\%$  (S.D.) and the splenic removal  $78 \pm 17\%$  (S.D.). The hepatic venous and the splenic venous blood were



## REMOVAL OF BACTERIA FROM BLOOD

TABLE III.  
Blood Levels of Bacteria and Percent Splanchnic Removal in Normal Rabbits.

Duration i.v. admin. baet. (min.)	7			8			10			12		
	C	H.V.	%Eh	C	H.V.	%Eh	C	H.V.	%Eh	C	H.V.	%Eh
8	141	89	36.9							261	216	17.2
15	140	120	14.3							214	151	29.4
20	160	117	26.9							400	185	53.8
29	172	153	11.0	386	214	44.6	480	393	18.2	283	214	24.1
36	190	156	17.9							257	212	17.5
43				701	496	29.2	490	417	14.9	348	242	30.5
50				699	642	8.2	647	505	21.9			
57				889	789	11.2	695	533	23.3			
64							867	640	26.2			
							904	803	11.2			

Duration i.v. admin. baet. (min.)	15			17			23		
	C	H.V.	%Eh	C	H.V.	%Eh	V.C.	H.V.	%Eh
8	651	520	20.1	360	338	6.1			
15	549	445	18.9	352	268	23.9	475	389	18.1
20	628	483	23.1				564	494	12.4
29	615	486	21.0	384	283	28.0	733	545	25.6
36	545	481	11.7	365	287	21.4	726	632	12.9
43	606	549	9.4	386	367	4.9	787	665	15.5
50	758	613	19.1						
57	993	828	16.6						

C.—Intracardiac.  
 H.V.—Hepatic venous.  
 V.C.—Vena caval.  
 %Eh—% splanchnic removal (C or V.C.—H.V.  $\times 100$ )

ermore the composition of the mineral matter found in bone, is related to that of the body fluids as would be expected from phase rule considerations for a solid solution.<sup>11-14</sup> However such physico-chemical principles do not explain why calcification takes place at specific sites and at a definite time in the life of the preosseous cells.

Calcification *in vitro* of the hypertrophic epiphyseal cartilage has served as a useful indicator of the cellular factors.<sup>2-4,7,8,15-21</sup> The appearance of new calcification depends on the functioning of the complete system essential for mineralization. Most of our knowledge of the "local factors" comes from such studies. Robison<sup>15,16,18</sup> by such *in vitro* studies indicated the importance of phosphatase. Gutman<sup>19</sup> has presented evidence that phosphorolative glycogenolysis is an integral part of the calcification process. However, one can demonstrate the presence of phosphatase and phosphorolative glycogenolysis in

tissues other than bone. Moreover Waldman<sup>21</sup> has shown that calcification *in vitro* can take place when phosphatase is inactivated. Furthermore, in rickets due to strontium, calcification *in vitro* is inactivated without affecting phosphatase activity.<sup>17,22</sup> Thus it can not be unequivocally stated that enzymes acting on organic phosphates are responsible for mineralization.

It occurred to us that by extending the studies of the injury to calcification in strontium rickets which is reversible *in vivo* and *in vitro*,<sup>17</sup> the role of a system handling calcium may be explored. (In addition we were stimulated by the statement of McLean<sup>23</sup> "Our search for a method of producing a reasonably complete but reversible inhibition of the local mechanism has so far failed.") This observation with strontium rickets and that of Robison<sup>24,25</sup> who caused mineralization of the hypertrophic epiphyseal cartilage *in vitro* by replacing  $\text{Ca}^{++}$  ions with  $\text{Sr}^{++}$  ions suggested that strontium competes with calcium for some constituent of the bone cell necessary for calcification. The more recent experiments using radioactive strontium as an indicator of calcium metabolism are in harmony with such a concept.<sup>26</sup>

It was reasoned that if rachitic bone sections (produced by the usual high calcium, low phosphorus diet) were shaken with  $\text{Sr}^{++}$  ions of high concentration, subsequent calcification would be inhibited by the competitive combination of strontium with a factor in the cell which is necessary for calcification. Moreover since inhibition did in fact take place, as

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<sup>8</sup> Niven, J. S. F., and Robison, R., *Biochem. J.*, 1934, **28**, 2237.

<sup>9</sup> Logan, M., *Physiol. Rev.*, 1940, **20**, 522.

<sup>10</sup> Huggins, C., *Physiol. Rev.*, 1937, **17**, 119.

<sup>11</sup> Eisenberger, S., Lehrman, A., and Turner, W. D., *Chem. Rev.*, 1940, **26**, 257.

<sup>12</sup> Sobel, A. E., Rockenmacher, M., and Kramer, B., *J. Biol. Chem.*, 1945, **159**, 159.

<sup>13</sup> Sobel, A. E., and Hanok, A., *J. Biol. Chem.*, 1948, **176**, 1103.

<sup>14</sup> Sobel, A. E., Hanok, A., Kirshner, H. A., and Fankuchen, I., *J. Biol. Chem.*, 1949, **179**, 205.

<sup>15</sup> Robison, R., *Biochem. J.*, 1923, **17**, 286.

<sup>16</sup> Robison, R., and Soames, K. M., *Biochem. J.*, 1924, **18**, 740.

<sup>17</sup> Sobel, A. E., Cohen, J., and Kramer, B., *Biochem. J.*, 1935, **29**, 2640.

<sup>18</sup> Robison, R., Significance of phosphoric esters in metabolism, 1932, New York University Press.

<sup>19</sup> Gutman, A. B., Warrick, F. B., and Gutman, E. B., *Science*, 1942, **95**, 461.

<sup>20</sup> Robison, R., *Ann. Rev. Biochem.*, Stanford University Press, 1936, **5**, 181.

<sup>21</sup> Waldman, J., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 262.

<sup>22</sup> Sobel, A. E., Cohen, J., and Kramer, B., *Biochem. J.*, 1935, **29**, 2646.

<sup>23</sup> McLean, F. C., Lipton, M. A., Bloom, W., and Barron, E. S. G., 14th Conference on metabolic aspects of convalescence, Symposium on bone metabolism, 1946, pp. 18, Josiah Macy, Jr. Foundation, New York.

<sup>24</sup> Robison, R., and Rosenheim, A. H., *Biochem. J.*, 1934, **28**, 684.

<sup>25</sup> Robison, R., and Rosenheim, A. H., *Biochem. J.*, 1936, **30**, 66.

<sup>26</sup> Norris, W. D., and Kisielski, W., *Symposia on Quantitative Biology*, Cold Spring Harbor, L. I., N. Y., 1948, **13**, 164.

cleared to the same degree. The absolute efficiency of these organs cannot be determined, as the blood flow per unit of tissue is not known in either instance. The percent removal of the bacteria remained high even when the arterial levels were maintained at 50,000 and 100,000 per ml. When one considers that the splanchnic circulation receives one-fifth of the cardiac output and that approximately 75% of the organisms are removed in a single circulation, it becomes clear why such a large number of bacteria is required to maintain a blood level of 100 organisms per ml.

The tissues of the hind limb showed no consistent ability to remove organisms from the circulating blood. The percent removed in a single circulation through the hind extremity was  $12 \pm 14\%$  (S.D.). No organisms were lost in circulation through the lungs of the dog.

In the present series of rabbits the total splanchnic removal of *Klebsiella pneumoniae* type B averaged  $20 \pm 10\%$  (S.D.). The effect of variation in the parasite on the rate of removal by the host will be discussed in a later communication.

Other studies<sup>1,5</sup> have shown that in the course of some experimental bacterial infections, the organisms are cleared partially or completely from the blood stream after a variable period of 5 to 48 hours, the variation depending apparently on the virulence of the infecting organism and the susceptibility of the host. After a period of several hours, the

blood culture may become positive again and may be associated with a progressively unfavorable clinical course leading to the death of the animal. The reason for the ineffective clearing of the blood stream in the later stage of the infection has not been determined. It may be that the removal mechanism is blocked by accumulation of organisms in the cells of the reticuloendothelial system, but this seems unlikely because the mechanism still functions efficiently at a level of 100,000 organisms per ml. The organisms may proliferate in the cells and so alter their physiologic functions. Observations on the sites from which the bacteria return to the blood stream and the state of the reticuloendothelial system at that time are needed.

*Summary.* 1. A method of constant intravenous infusion of bacteria combined with the determination of bacterial counts in the circulation at various sites by venous catheterization is described, which provides a means of determining the site and of quantitating the rate of removal of bacteria from the blood stream of the intact animal.

2. The total canine splanchnic removal of hemolytic *Micrococcus aureus* averaged  $74 \pm 16\%$  (S.D.); splenic removal,  $78 \pm 17\%$  (S.D.). No organisms were lost in circulation through the lungs.

3. The total rabbit splanchnic removal of encapsulated *Klebsiella pneumoniae* type B averaged  $20 \pm 10\%$  (S.D.).

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## The Reversible Inactivation of Calcification *in vitro*.<sup>\*</sup> (17334)

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Some aspects of normal calcification can be explained by physico-chemical concepts. For example, new calcification depends on the concentration of calcium and phosphate

ions in excess of a critical product.<sup>1-10</sup> Furth-

<sup>1</sup> Howland, J., and Kramer, B., *Tr. Am. Pediat. Soc.*, 1922, **34**, 204.

<sup>2</sup> Shipley, P. G., *Bull. Johns Hopkins Hosp.*, 1924, **35**, 304.

<sup>3</sup> Shipley, P. G., Kramer, B., and Howland, J., *Biochem. J.*, 1926, **20**, 379.

<sup>\*</sup> Supported in part by a grant from the Williams-Waterman Fund for the Combat of Dietary Diseases, Research Corporation, New York.

TABLE II.

Reversible Inactivation of Calcification *in vitro* with  $\text{CuCl}_2$  in the Presence of  $\text{CaCl}_2$ .

Control*		Inactivation—2 hr shaking		Reactivation—1 hr shaking	
No treatment					
Degree of calcification†	Solution, mE/L		Degree of calcification‡	Solution, mE/L	
	$\text{CaCl}_2$	$\text{CuCl}_2$		$\text{CaCl}_2$	Degree of calcification‡
2(+ + + +)	150	0.0	4(+ + + +), 4(+ + + +)		
2(+ + + +)	150	0.1	4(+ + + +), 4(+ + + +)	150	4(+ + + +), 4(+ + + +)
2(+ + + +)	150	0.3	4(+ + + +), 4(+ + + +)	150	4(+ + + +), 4(+ + + +)
3(+ + + +)	150	0.5	1(+ + + +), 1(+ + + +)	150	4(+ + + +), 4(+ + + +)
3(+ + + +)	150	0.5	1(+), 0(0)	150	4(+ + + +), 4(+ + + +)
2(+ + + +)	150	0.5	1(+), 1(+)	150	4(+ + + +), 4(+ + + +)
2(+ + + +)	150	0.75	2(+ + + +), 2(+ + +)	150	4(+ + + +), 4(+ + + +)
2(+ + + +)	150	1.0	1(+), 1(+)	660	4(+ + + +), 3(+ + + +)
	150	1.0	1(+), 2(+ + +)	150	4(+ + + +), 3(+ + + +)
	150	1.0	3(+ + + +), 4(+ + + +)	150	4(+ + + +), 4(+ + + +)
	150	1.0	0(0), 0(0)	150	4(+ + + +), 4(+ + + +)
	150	2.0	0(0), 0(0)	150	4(+ + + +), 4(+ + + +)
	150	3.0	0(0), 0(0)	660	3(+ + + +), 1(+)
	150	3.0	0(0), 0(0)	150	4(+ + + +), 1(+ + + +)
	150	4.0	0(0), 0(0)	150	1(+), 1(+)
	150	5.0	0(0), 0(0)	150	0(0), 0(0)
	150	6.0	0(0), 0(0)	150	1(+), 1(+)
	150	10.0	0(0), 0(0)	150	0(0), 0(0)

\* Specimens incubated in calcifying solution for 18-24 hr.

Calcifying solution contains: 0.7 eq/L NaCl

0.05 eq/L KCl

0.22 eq/L  $\text{NaHCO}_3$  $\text{Ca} \times \text{P}$  product = [10 mg % Ca] [5 mg % P] = 50, unless otherwise noted.

† The degree of calcification is indicated as follows: 0(0) no calcification; 1(+) trace; 1(++) broken thin line; 1(+++) almost complete thin line across the provisional zone; 1(++++) complete thin line across the provisional zone; 2(++++) heavy line across the provisional zone including the primary tongues of cartilage; 3(++++) heavy line across the provisional zone including the primary and secondary tongues of cartilage; 4(++++) practically complete calcification of the metaphysis.

‡ Calcifying solution same as that used previously (Table I).

 $\text{Ca} \times \text{P}$  product kept constant at  $10 \times 5.0 = 50$ .

shown in Table I, it was further reasoned that the reverse reaction should take place, by shaking with  $\text{Ca}^{++}$  ions of high strength. This actually turned out to be the case as shown in Table I. The calcification was usually more extensive than in the untreated control sections. Further experiments showed that similar reversible inactivation is possible by preliminary shaking with a high concentration of NaCl, NaCl of physiological strength adjusted to a pH of 3, and to a smaller degree with physiological saline adjusted to a pH of 9.6.

These experiments demonstrate that it is possible to reversibly inactivate calcification *in vitro* by electrolytes but do not prove that it takes place by competitive retardation. One can also explain these results as due to the simple removal of  $\text{Ca}^{++}$  ions from the cell when shaking with calcium free solutions. In the

reactivation step, these  $\text{Ca}^{++}$  ions are replaced so that subsequent calcification can proceed. That reversible inactivation does not depend on the mere removal of  $\text{Ca}^{++}$  ions was shown in the following experiments. When rachitic bone sections were shaken with 150 mE/L of  $\text{CaCl}_2$  in the presence of as little as 0.5 mE/L of  $\text{CuCl}_2$ , calcification was inactivated as shown in Table II. When the section was subsequently shaken with the same concentration of  $\text{CaCl}_2$  in the absence of the  $\text{CuCl}_2$ , reactivation took place which again was more extensive than in the untreated control sections.

These experiments indicate that  $\text{Cu}^{++}$  ions combined reversibly with a necessary portion of the calcifying system. They further suggest that an important step in calcification is the combination of calcium with some constituent of the bone cell, probably part of an

TABLE I.  
Reversible Inactivation of Calcification *in vitro* by  $\text{SrCl}_2$  and  $\text{NaCl}$  Solutions.

Control* No treatment	Inactivation			Reactivation		
	Degree of calcification†	Solution mE/L	Hr of shaking	$\text{Ca} \times \text{P}^\ddagger$ product	Hr of shaking	$\text{Ca} \times \text{P}$ product
						Degree of calcification†
		$\text{SrCl}_2$ -150	2	50	1	50
		$\text{SrCl}_2$ -150	2	50	1	50
	2(+ + + + +)	$\text{CaCl}_2$ -666	2	45	1	45
	2(+ + + + +)	$\text{SrCl}_2$ -666	2	45	1	45
		$\text{NaCl}_2$ -666	2	45	1	45
	1½(+ + + + +)	$\text{CaCl}_2$ -666	3	40	1	40
		$\text{SrCl}_2$ -666	3	40	1	20
	1½(+ + + + +)	$\text{NaCl}$ -666	3	40	1	40
	2(+ + + + +)	$\text{CaCl}_2$ -666	3	40	1	20
	0(0)⁵			20		
	2(+ + + + +)	$\text{CaCl}_2$ -666	3	45	1	45
		$\text{NaCl}$ -167	3	45	1	0
		$\text{SrCl}_2$ -167	3	45	1	0
	2(+ + + + +), 2(+ + + + +)	Saline ad- justed to pH3 with $\text{HCl}$	½	45	1	45
		Saline ad- justed to pH9.6 with $\text{NaOH}$	½	45	1	45

\* Specimens incubated in calcifying solution for 18-24 hr.

Calcifying solution contains: 0.7 eq/L  $\text{NaCl}$

0.05 eq/L  $\text{NaCl}$

0.22 eq/L  $\text{NaHCO}_3$

$\text{Ca} \times \text{P}$  product = 10 mg %  $\text{Ca} \times 5$  mg %  $\text{P}$  unless otherwise noted.

† The degree of calcification is indicated as follows: 0(0) no calcification; 1(+) trace; 1(+ + +) broken thin line; 1(+ + + +) almost complete thin line across the provisional zone; 1(+ + + + +) complete thin line across the provisional zone; 2(+ + + + +) heavy line across the provisional zone including the primary and secondary tongues of cartilage; 3(+ + + + +) heavy line across the provisional zone including the primary and secondary tongues of cartilage; 4(+ + + + +) practically complete calcification of the metaphysis.

‡  $\text{Ca} \times \text{P}$  products are obtained by keeping  $\text{Ca}$  concentration constant ( $\text{Ca} = 10$  mg %) and varying  $\text{P}$  concentration thus:  $\text{Ca} \times \text{P} = 10 \times 0 = 0$ ;  $\text{Ca} \times \text{P} = 10 \times 2 = 20$ ;  $\text{Ca} \times \text{P} = 10 \times 4.5 = 45$ ; and  $\text{Ca} \times \text{P} = 10 \times 5.0 = 50$ .

§ Physiological saline was used;  $\text{NaCl} = 0.145$  eq/L.

||  $\text{Ca} \times \text{P}$  product for this control = 20.

TABLE I.  
Influence of Alloxan Diabetes on Cholesterol Atheromatosis in Rabbits.  
Group A Control Group—Basal diet and cholesterol in olive oil.

Rabbit No.	Duration of diabetes, diet, days	Terminal blood sugar, mg/100 cc	Terminal blood cholesterol, mg/100 cc	Body wt		Atheromata	
				Initial, g	Final, g	Gross	Micro.
48-6	38			910	1110	0	0
48-21	40	132	576	1150	1610	0	0
48-25	41	110	864	823	1160	0	+
48-22	48	120	864	1000	1640	++	++
48-20	49			1080	1790	+	+
48-4	58		1120	1060	1535	0	+
48-8	72		1025	1500	2225	+++	+++
48-10	72		1425	1425	1755	++	++
48-15	72		837	1961	2200	+++	+++
Avg	54	121	959	1212	1670		
Avg of control values		120	141		1212		
% change		+1	+580		+38		

sugar determination was performed. Blood cholesterol values were determined on each animal of this group before alloxan injection and before sacrificing according to the method of Bloor, Pelkan and Allen.<sup>2</sup> Those rabbits which manifested a blood sugar of 200 mg% or higher were accepted as diabetic. A total of 8 rabbits satisfying this criterion and showing persistent glycosuria for the duration of the experiment were thus obtained.

Nine rabbits of similar strain, age, and original body weight were used as a control group. Blood sugar values were determined on 3 of these animals before beginning the diet and before sacrificing, and blood cholesterol levels were determined on all except 2 animals at the same times.

A third group consisted of 3 rabbits which had received 2 injections of alloxan and had manifested a temporary glycosuria and hyperglycemia, but the urines of which later became sugar free.

For the cholesterol enriched diet, Pfansteil cholesterol suspended in olive oil to a concentration of 6% was added to Rockland Rabbit Ration in such amounts that each rabbit received between 0.3 and 0.6 g per day.

All rabbits of the 3 groups were then fed this cholesterol enriched diet and animals from each group were sacrificed at various intervals after commencement of the diet. Complete

autopsies were performed, with especial attention being paid to the heart, aorta, and great vessels. The presence of atheromata in these organs is summarized in the tables. It may be mentioned here that the pathological changes with respect to the other organs were similar to those previously described for alloxan diabetes and cholesterol atheromatosis separately.

*Experimental observations.* Table I summarizes the data on the control group. It is seen that there was a consistent increase in blood cholesterol values, the increase roughly paralleling the duration of the diet, and the average increase being approximately seven fold. The weight increase was also consistent. The item of chief interest is the fact that in all animals fed the diet 41 days or longer, there was gross or microscopic evidence of atheromata, the severity of the lesions roughly corresponding to the duration of the diet. The atheromata were in all respects similar to those previously described in this condition.

In Table II are seen the summarized data on the diabetic animals. Among these the elevation in blood cholesterol was very irregular, but the average is higher than the average for Group A, and in 3 cases it is considerably higher than that of any single rabbit in Group A. All of these rabbits except two (48-26 and 48-27) lost weight. Much to our astonishment, when these animals were autopsied, only one showed any evidence of

<sup>2</sup> Bloor, W. R., Pelkan, K. F., and Allen, D. M., *J. Biol. Chem.*, 1922, 52, 191.

enzyme system. In a calcium free medium, calcium is removed and is probably replaced by one of the competing ions.  $\text{Cu}^{++}$  ions may compete so avidly for this local constituent that they preferentially combine with this factor even in the presence of large amounts of  $\text{Ca}^{++}$  ions. On shaking with  $\text{CaCl}_2$  solution free of the competing ions, the formation of the calcium complex is favored as an initial step to calcification.

*Summary.* It was possible to demonstrate the reversible inactivation of calcification *in*

*vitro* of the hypertrophic epiphyseal cartilage. When rachitic bone sections are shaken with strontium chloride, sodium chloride, calcification *in vitro* is inhibited. On subsequent shaking with calcium chloride calcification *in vitro* takes place. In addition, inactivation takes place with  $\frac{1}{2}$  mE of cupric chloride in the presence of 150 mE of calcium chloride. On subsequent shaking with 150 mE of calcium chloride (in the absence of cupric chloride) reactivation takes place.

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### The Influence of Alloxan Diabetes on Cholesterol Atheromatosis in the Rabbit.\*† (17335)

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It has long been suspected, largely on the basis of statistical studies, that humans suffering from diabetes mellitus develop arteriosclerosis not only in greater numbers than do non-diabetics, but also develop it earlier. However, conclusive experimental evidence that diabetes enhances arteriosclerosis has been lacking. It seemed to us that it might be instructive to test the influence of alloxan diabetes in the rabbit, despite the fact that the relationship of both experimental entities to their human counterparts are poorly understood.

*Methods.* The general plan of the experiment was as follows: 3 groups of rabbits, one group of which had been made diabetic with alloxan, were fed a cholesterol enriched diet; and animals from each group were examined at intervals after commencement of the diet

to see if there was any difference in the time of onset or character of the vascular lesions in the different groups. Young Dutch rabbits weighing from 900 to 1400 g were given intravenous injections of alloxan monohydrate (Eastman) amounting to 80 to 100 mg per kilo of body weight daily for 2 consecutive days. Animals that were not diabetic on the third day were given similar injections on the third and fourth successive days. Although this method had been found in previous experiences with alloxan to be very effective, nevertheless in this instance a large number of animals died either during the course of the injections or within a week thereafter. Only approximately one half of the animals developed diabetes and survived long enough to be of value in the experiment.

Blood sugar determinations were performed by the method of Somogyi,<sup>1</sup> using a Klett-Summerson photoelectric colorimeter, both before and after alloxan injection. During the period of feeding the diet, the urines of the diabetic group were tested periodically with Benedict's qualitative reagent, and just before each animal was sacrificed, another blood

\* This work was aided by a grant from The John and Mary R. Markle Foundation.

† Since preparation of this manuscript the results of G. L. Duff and G. C. McMillan (abstracted in *Am. Heart J.*, 1948, 36, 469) have come to our attention. These results confirm and extend those presented in this paper in showing that alloxan diabetes inhibits experimental cholesterol atherosclerosis in the rabbit.

<sup>1</sup> Somogyi, M., *J. Biol. Chem.*, 1945, 160, 61.

no atheromata and no reports of finding atheromata in alloxan diabetic rabbits have appeared in the literature.

In discussion we would like to mention one observation concerning the food intake of these animals. It was noted soon after the beginning of the experiment that, contrary to previous experiences with regular diets in which diabetic animals consumed much more food than normal rabbits, these diabetic rabbits did not eat the oily diet nearly so enthusiastically as did the controls. A greater amount of cholesterol had to be added per unit weight of ration to insure that each rabbit received at least 0.3 g daily. Facilities did not permit us to determine the exact daily consumption of each rabbit. The control animals, therefore, may have received somewhat more cholesterol per day than did the diabetic animals, but it is certain that they did not receive more than twice as much. It is our opinion that the difference in intake was not sufficiently great to account for the very

great difference in time of onset of atheromata.

Whatever the mechanism, and despite the above mentioned criticisms, we feel that these findings clearly indicate that under the conditions of this experiment alloxan diabetes retards in time and degree the development of cholesterol atheromatosis in the rabbit, and that it represents a conclusive demonstration that hypercholesterolemia of itself does not lead to the formation of atheromata.

*Summary.* Alloxan diabetic rabbits, when fed a cholesterol enriched diet, developed blood cholesterol levels much higher than did normal rabbits fed the same diet. Atheromata appeared consistently in controls (and also in alloxan injected, nondiabetic rabbits) after 41 days on this diet, but only to a slight degree in one diabetic rabbit fed the same diet for 98 days. There was no qualitative difference in the character of the lesions in the 3 groups.

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## Studies on the Pathogenesis of Experimental Necrotizing Arteritis.\* (17336)

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Previous studies from this laboratory<sup>1-3</sup> have shown that acute necrotizing arteritis can be produced with regularity in dogs by feeding a specified high fat diet for a period of 8 weeks or longer then sacrificing them through renal damage. Three factors, time, diet and renal damage, seem to be essential in the production of these lesions.

Methods have been published in detail previously.<sup>2</sup> Briefly these consist of feeding healthy adult dogs a high fat diet for a period

of 8 weeks or longer, then sacrificing them through renal damage. The active "dietary factor" is found in cod liver oil but is apparently not unique to cod liver oil. The oil may be added to a kennel diet of unselected table scraps or to a "standard diet", consisting of calves liver (raw wet weight), 32 parts; cane sugar, 25 parts; corn starch, 25 parts; butter, 12 parts and cod liver oil, 6 parts, with equal results. Renal damage has been produced by any one of 3 ways: 1) uranium nitrate subcutaneously; 2) mercuric chloride intravenously; and 3) bilateral nephrectomy; all equally effective.

In all experiments to date, renal damage has been essential. To determine whether or not damage to tissues and organs other than the kidney would precipitate the arterial le-

\* This work was aided by a grant from The John and Mary R. Markle Foundation.

<sup>1</sup> Holman, R. L., *Proc. Soc. Exp. Biol. and Med.*, 1947, 66, 307.

<sup>2</sup> Holman, R. L., and Swanton, M. C., *Proc. Soc. Exp. Biol. and Med.*, 1946, 63, 87.

<sup>3</sup> Holman, R. L., *So. Med. J.*, 1949, 42, 108.



## ALLOXAN DIABETES AND CHOLESTEROL ATHEROMATOSIS

TABLE II.  
Influence of Alloxan Diabetes on Cholesterol Atheromatosis in Rabbits.  
Group B Diabetic Group—Same as control plus alloxan.

Rabbit No.	Duration of diabetes, diet, days		Terminal blood sugar, mg/100 cc	Terminal blood cholesterol, mg/100 cc	Body wt		Atheromata	
					Initial, g	Final, g	Gross	Micro.
47-7	22	18	365	503	940	763	0	0
47-12	20	20	297		1060	550	0	0
47-11	31	31	279	2000	1213	1175	0	0
47-8	41	41	268	665	1360	550	0	0
48-26	43	41	205	960	1000	1300	0	0
48-27	48	48	190	349	692	1380	0	0
48-11	68	65	300	2000	1220	650	0	0
47-10	98	98	319	3820	1010	910	+	+
Avg	47	46	278	1287	1062	910		
Avg of control values			117	132		1062		
% change			+138	+875		-14		

TABLE III.  
Influence of Alloxan Diabetes on Cholesterol Atheromatosis in Rabbits.  
Group C—Alloxan injected—non-diabetic.

Rabbit No.	Duration of diabetes, diet, days		Highest blood sugar, mg/100 cc	Terminal blood cholesterol, mg/100 cc	Body wt		Atheromata	
					Initial, g	Final, g	Gross	Micro.
48-24	?	40	180	368	1070	1530	+	+
48-28	?	52	230	368	910	1570	+++	++
48-3	?	69	157	672	985	1650	++	++
Avg		54	189	469	988	1583		
Avg of control values			120	140		988		
% change			+58	+235		+60		

TABLE IV.  
Influence of Alloxan Diabetes on Cholesterol Atheromatosis in Rabbits.  
Summary of data on all rabbits fed diet for 40 days or longer.

Group	No. with atheromata	
	No. in group	Percent positive
A. Control	7/8	88
B. Diabetic	1/5	20
C. Alloxan injected, non-diabetic	3/3	100

atheromata—and this rabbit (47-10) had been fed the diet for 98 days.

In Table III are the data on the alloxan-injected, non-diabetic animals, in which it is seen that they behaved as did the controls in Group A.

Table IV summarizes the incidence of atheromata in all rabbits fed the high cholesterol diet for 40 days or longer. These figures become even more impressive when one recalls that the one diabetic animal had de-

veloped only minimal lesions after 98 days on the diet.

*Discussion.* In criticism it may be pointed out that the number of animals is very small and that there were no diabetic animals kept as controls. These defects were due to the difficulties encountered in inducing alloxan diabetes. However, with respect to the latter point, in previous experiences by one of the authors with alloxan diabetic rabbits several animals kept for as long as 11 months showed

tive day, each had about 300 cc of clear fluid in the peritoneal cavity; but neither dog had arterial lesions. Histological study of the kidneys showed early hemorrhage and necrosis about the angles of the calyces and early hydronephrosis. The collecting tubules were moderately dilated but the remaining tubules were remarkably well preserved.

*Discussion.* These experimental observations reaffirm the importance of the kidney in the pathogenesis of arterial lesions. All of our results to date indicate that some derangement of renal function is prerequisite to the development of arterial lesions. Other workers<sup>4-6</sup> have produced arterial lesions with various types of renal insufficiency, but none of these workers has found it necessary to control the diet of his experimental animals. Whereas, in our experience, "standard renal insufficiency" alone resulted in "typical arterial lesions" in only 5 of 130 dogs (4%), the combination of "standard high fat diet" and "standard renal insufficiency" resulted in "typical arterial lesions" in 36 to 40 dogs (90%). This discrepancy between our results incriminating a "dietary factor" and the results of others ignoring a "dietary factor" may, in part, be related to the definition of "typical arterial lesions", for we do not consider some of the hemorrhagic lesions illustrated in previous publications<sup>4,5</sup> "typical" of our experimental lesions which are predominately necrotizing in character and only occasionally associated with gross hemorrhage.

If, as the above results indicate, "standard high fat diet" and "standard renal insufficiency" are *both* necessary for the production of "typical arterial lesions," the problem simmers down to what role the kidney plays in the metabolism of the potentially noxious fatty substance or substances contained in the specified high fat diet. During the 2 months or more of high fat feeding, the kidneys—and

especially the epithelial cells lining the loops of Henle along with those of the distal portion of the proximal convoluted tubules—become distended with sudanophilic material. This condition is still compatible with normal life, for the high fat diet can be fed indefinitely and no predictable changes in the vascular system are ever observed unless the kidneys are damaged. Anytime after 2 months of such a diet "standard renal insufficiency" is regularly followed by "typical arterial lesions". It can be surmised that the intact kidney elaborated something necessary for the metabolism of the noxious lipids and that when the kidney is severely damaged the noxious lipids (or metabolic by-products thereof) pile up to "explosive" levels as manifested by the arterial lesions.

The factor common to all the methods used for the production of "standard renal insufficiency" that have resulted in "typical arterial lesions" is massive "damage" to the proximal convoluted tubules in a relatively short period of time (massive coagulative necrosis with uranium nitrate and mercuric chloride and mass exclusion in the case of bilateral nephrectomy). The experiments reported in this paper show that severe damage to tissues and organs other than the kidneys does not precipitate arterial lesions in properly fed dogs and that bilateral ureteral ligation—despite degrees of azotemia, phosphatemia, acidosis, and "uremia" corresponding to those produced by "standard renal insufficiency"—is likewise ineffective in precipitating arterial lesions in properly fed dogs.

The simplest explanations that we have been able to formulate for these unanticipated findings are: (1) the proximal convoluted tubules elaborate something (lipase?) necessary for the proper utilization of certain lipid substances or (2) the integrity of the proximal convoluted tubules is necessary for the neutralization of certain toxic substances (amines?). These hypotheses are based in large part upon the fact that six days after bilateral ureteral ligation the epithelium lining the proximal convoluted tubules is fairly well preserved whereas six days after "standard renal insufficiency" this epithelium appears to

<sup>4</sup> Wintenz, M. C., Myon, E., Walters, L. L., and Katzenstein, R., *Yale J. Biol. and Med.*, 1940, **12**, 623.

<sup>5</sup> Wintenz, M. C., and Katzenstein, R., *Yale J. Biol. and Med.*, 1940, **13**, 15.

<sup>6</sup> Goldblatt, H., *Harvey Lectures*, 1937-38, **33**, 237.

TABLE I.  
Studies on the Pathogenesis of Experimental Necrotizing Arteritis.

Group	Dog No.	Period on standard diet before injury, wks	Type of injury	Total dose	Survival interval, days	Terminal NPN	Arterial lesions
Group A	47-41	10	Chloroform anesthesia	28 hr	20	50	0
Chloroform Injury to liver	47-46	9	"	10 hr	6	68	0
	47-50	9	"	13 hr	9	50	0
	47-74	13	"	12 hr	7	5*	0
Group B	47-73	12	Turpentine	32 cc	18	37*	0
Suppuration	47-64	13	Peritonitis		21	12*	0
	48-94	14	Cellulitis		3	58*	0
Group C	49-34	9	Bilateral ureteral ligature		6	255*†	0
Bilateral ureteral ligature	49-35	9	"		6	168*‡	0

\* Blood urea nitrogen instead of non-protein nitrogen.

† Terminal blood serum phosphorus 21 mg per 100 ml.

‡ Terminal blood serum phosphorus 13 mg per 100 ml and terminal blood carbon dioxide combining power 38 volumes %.

sions in properly fed dogs, the following two series of experiments were undertaken:

1) *Chloroform injury to the liver.* Four properly fed dogs (Table I, Group A) were subjected to light chloroform anesthesia for periods totalling 10-28 hours in 6-20 days. Marked liver injury was indicated by the appearance of jaundice and was confirmed by histological study of the liver. None of these 4 dogs showed gross or microscopic lesions in the arterial system.

2) *Suppuration* (Table I, Group B). One dog (47-13) was given repeated subcutaneous injections of turpentine for a total dose of 32 cc in 18 days. Two of the sterile abscesses broke down and each discharged about 100 cc of sanguinopurulent material. The total white blood cell count rose progressively to 45,500. At the time of autopsy there were 12 subcutaneous abscesses estimated to contain a total of 400 cc of similar purulent material but no arterial lesions.

The experimental induction of intestinal obstruction was attempted in two other dogs but neither operation accomplished its intended purpose. Instead, in one dog (47-64) the ligature (umbilical tape about 1 foot above the ileocecal valve) cut through the bowel and the dog died with generalized peri-

tonitis on the 21st day. In the second attempt (Dog 48-94) the small intestine about 2 feet below the ligament of Treitz, was divided between ligatures and the closed proximal end was sutured into the subcutaneum of the abdominal wall. This proximal stump "blew out" and the dog died on the 3rd day with massive fecal contamination of the subcutaneum (associated with cellulitis and fat necrosis) that extended from the hypogastrium to below the inguinal ligament. Despite the massive necrosis and suppuration no arterial lesions were found in either of these two dogs.

Since severe renal damage has been a prerequisite for the arterial lesions the following experiments were undertaken in an attempt to define more accurately what phase or part of "renal insufficiency" was responsible for the subsequent anatomical changes in the arterial system.

*Bilateral ureteral ligations.* Under nembu-tal anaesthesia both ureters of 2 properly fed dogs (Table I, Group C) were doubly ligated and divided between ligatures at a level midway between the uretero-pelvic and uretero-vesical junctions. Each dog stopped eating on the 2nd post-operative day and each showed a progressive azotemia, acidosis, phosphatemia, and "uremia". At autopsy on the 6th post-opera-

TABLE I.  
Sexual Behavior of Two Groups of Male Rats Tested in Opposite Phases of Diurnal Light Cycle.

Measure of sexual performance	Group I (N = 14)		Group II (N = 13)	
	Tested in dark phase	Tested in light phase	Tested in light phase	Tested in dark phase
% of group copulating	85.7	71.4	23.1	92.3
% of group ejaculating	78.6	64.3	7.7	92.3
Avg frequency of copulation*	9.3	8.4	8.3	10.6
Avg frequency of ejaculation*	1.2	1.0	1.0	1.3
Avg seconds delay before first copulation	46.0	66.0	66.0	40.0

\* Average frequencies based on performance of animals showing the response at least once in the test.

marized in Table I, which is based upon the average performance of each group during tests 2 and 4. Comparable group differences were apparent in the first and third tests, but it seems preferable to restrict our attention to behavior occurring in the second test of each series because this allows for preliminary adjustment to handling and testing during the particular phase of the light cycle that happens to be involved. The 14 animals in Group I were observed during the dark phase of the cycle in tests 1 and 2, whereas for the 13 males in Group II the first 2 tests occurred in the middle of the lighted portion of the 24-hour period. Group I was by far the most active sexually. Rats tested during the dark phase of the cycle were more likely to copulate and to ejaculate than animals placed with females in the light phase. And when coital behavior appeared it involved a higher frequency of intromissions and ejaculations in the case of the males tested during their "night." Considering only those animals that showed mating responses, the average latency, or delay preceding initiation of sexual contact was shorter for Group I than for Group II.

The time of testing was shifted by 12 hours during the third and fourth tests, and as a result the performance of animals in Group II improved appreciably. The proportion of the group showing copulatory responses increased by 69.2%, and the number reaching the point of ejaculation rose from 7.7 to 92.3%. The mean frequency of com-

pleted copulations and ejaculations increased, and the average duration of latencies was reduced. There is little doubt that most if not all of the increased sexual activity was due to changing the time of testing from the light to the dark phase of the diurnal light cycle.

Theoretically it might have been anticipated that the performance of Group I would deteriorate during the third and fourth tests, since at this time these animals were being tested during their "day." Some decrease in activity did occur and all changes were in the expected direction, but the scores remained well above those recorded for Group II during the first 2 tests. When Group I rats were shifted from "night" to "day" tests, the proportion of animals copulating at least once decreased, as did the percentage of the group achieving ejaculation. The average frequencies of copulations and ejaculations were similarly reduced, and the mean delay before initiation of sexual responses grew appreciably longer.

*Discussion.* Comparisons of the performance of the 2 experimental groups in test 2 fully confirms the hypothesis that male rats are sexually more active during the dark phase of their light-dark cycle. Care was taken to hold testing conditions constant for all males, and the females used as stimulus objects for the two groups were equally active and receptive. The pronounced change in the behavior of Group II animals when their tests were shifted from the light to the dark phase of

be almost completely destroyed. If these experimental findings are confirmed and if our reasoning is valid, the identification of these hypothetical substances (lipases?, amines?) might help clarify the time-honored relation of the kidney to arterial lesions.

*Summary.* Previous studies have shown that the combination of "standard high fat diet" plus "standard renal damage" resulted in typical arterial lesions in 36 of 40 dogs (90%). The data presented in this paper indicate: (1) "Standard high fat diet" plus damage to organs and tissues other than the kidney (chloroform injury to the liver, turpentine ab-

scesses of subcutaneum, and bacterial infections) failed to produce arterial lesions and (2) "Standard high fat diet" plus renal insufficiency produced by bilateral ureteral ligation also failed to produce arterial lesions. These studies re-emphasize the importance of the kidney in the pathogenesis of necrotizing arteritis and indicate that azotemia *per se* is not the "renal factor". They suggest the possibility that the epithelium of the proximal convoluted tubules elaborates one or more substances necessary for the proper utilization of certain lipid substances.

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## Diurnal Variations in the Mating Behavior of Male Rats.\* (17337)

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Female albino rats usually come into estrus and display their most active mating behavior during the night;<sup>1</sup> and animals maintained under artificial illumination with a reversed light-dark cycle become receptive during the solar day.<sup>2</sup> The present investigation tested the hypothesis that a comparable rhythm of sexual responsiveness occurs in males of the same species.

*Methods.* Twenty-seven adult, virgin males of the Sprague-Dawley strain were divided into 2 groups that were kept in different halves of a windowless room which was bisected by a black curtain. From 11 P.M. to 11 A.M. one half of the room was dark and the other was illuminated by 2 100 watt bulbs. From 11 A.M. to 11 P.M. the lighting conditions in the two halves of the room were reversed. For approximately 3 weeks before testing began and throughout the remainder of the experiment all animals lived under identical conditions save for the fact that for Group I it was

"day" from 11 P.M. to 11 A.M. and for Group II this same period was "night."

The first 2 mating tests were conducted between the hours of 3 P.M. and 7 P.M. The tests occurred, therefore, in the middle of the "night" as far as Group I was concerned, and in the middle of the "day" for Group II. The third and fourth tests were made from 3 A.M. to 7 A.M., thus reversing the temporal placement employed in tests 1 and 2. Mating tests for each male were spaced 3 days apart to allow for complete recovery from effects of sexual exercise. The stimulus animals were spayed, Sprague-Dawley females brought into heat by injection of estrogen and progesterone.<sup>1</sup> Methods of testing sexual behavior were the same as those employed by the senior author in previous studies.<sup>3</sup> A test lasted for 10 minutes if there was no coital behavior, and for 10 minutes from the time of the first complete or incomplete copulation if either response occurred.

*Results.* The experimental results are sum-

\* This investigation was supported by a grant from the Committee for Research in Problems of Sex, National Research Council.

<sup>1</sup> Ball, J., *Comp. Psychol. Monogr.*, 1937, 14, 1.

<sup>2</sup> Beach, F. A., *J. Comp. Psychol.*, 1938, 20, 355.

† The hormone preparations were generously supplied by Dr. Edward Henderson of Schering Corporation, Bloomfield, N. J.

<sup>3</sup> Beach, F. A., *J. Exp. Zool.*, 1944, 97, 249.

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	Tested in dark phase	Tested in light phase	Tested in light phase	Tested in dark phase
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marized in Table I, which is based upon the average performance of each group during tests 2 and 4. Comparable group differences were apparent in the first and third tests, but it seems preferable to restrict our attention to behavior occurring in the second test of each series because this allows for preliminary adjustment to handling and testing during the particular phase of the light cycle that happens to be involved. The 14 animals in Group I were observed during the dark phase of the cycle in tests 1 and 2, whereas for the 13 males in Group II the first 2 tests occurred in the middle of the lighted portion of the 24-hour period. Group I was by far the most active sexually. Rats tested during the dark phase of the cycle were more likely to copulate and to ejaculate than animals placed with females in the light phase. And when coital behavior appeared it involved a higher frequency of intromissions and ejaculations in the case of the males tested during their "night." Considering only those animals that showed mating responses, the average latency, or delay preceding initiation of sexual contact was shorter for Group I than for Group II.

The time of testing was shifted by 12 hours during the third and fourth tests, and as a result the performance of animals in Group II improved appreciably. The proportion of the group showing copulatory responses increased by 69.2%, and the number reaching the point of ejaculation rose from 7.7 to 92.3%. The mean frequency of com-

pleted copulations and ejaculations increased, and the average duration of latencies was reduced. There is little doubt that most if not all of the increased sexual activity was due to changing the time of testing from the light to the dark phase of the diurnal light cycle.

Theoretically it might have been anticipated that the performance of Group I would deteriorate during the third and fourth tests, since at this time these animals were being tested during their "day." Some decrease in activity did occur and all changes were in the expected direction, but the scores remained well above those recorded for Group II during the first 2 tests. When Group I rats were shifted from "night" to "day" tests, the proportion of animals copulating at least once decreased, as did the percentage of the group achieving ejaculation. The average frequencies of copulations and ejaculations were similarly reduced, and the mean delay before initiation of sexual responses grew appreciably longer.

*Discussion.* Comparisons of the performance of the 2 experimental groups in test 2 fully confirms the hypothesis that male rats are sexually more active during the dark phase of their light-dark cycle. Care was taken to hold testing conditions constant for all males, and the females used as stimulus objects for the two groups were equally active and receptive. The pronounced change in the behavior of Group II animals when their tests were shifted from the light to the dark phase of

the artificial light cycle is added proof of the greater sexual potency during the "nocturnal" hours.

Female rats tend to come into heat during the night because of the associated rhythm of secretion of gonadal hormones. This rhythm in turn reflects the cyclic secretion of gonadotrophic hormones by the anterior pituitary, the functions of which are influenced by periodic increase and decrease in the amount of environmental illumination as well as concomitant changes in external temperature. Regular fluctuations in hypophyseal activity also affect the secretory behavior of the thyroid and probably of still other endocrine glands. As a result, the albino rat displays a diurnal rhythm of energy metabolism which begins to increase at approximately noon, reaches a maximum around 9 P.M. and progressively declines to the next noon. If rats are placed under constant illumination and fed at frequent intervals, the metabolic rhythm is abolished.<sup>4</sup>

There is no reason to believe that the rat testis secretes androgen at significantly different rates during different times of day, and even if it did so the behavioral response to alteration in the concentration of testicular hormone is too slow to account for the diurnal variations that characterize the male's mating performance. The heightened sexual excitability shown by male rats during the dark phase of the illumination cycle probably is due to the nocturnal increase in basal metabolism and to a consequent increase in responsiveness to all kinds of exteroceptive stimulation.

The sexual activity of males in Group I decreased only slightly when the time of testing was changed from the dark to the light phase of the 24-hour period. In all probability the continued high level of responsiveness was due to the effects of the first two tests

when these animals were mated during their "night." In tests 1 and 2 the majority of Group I males gained a considerable amount of sexual experience. They became accustomed to receiving receptive females in the observation cage and achieved orgasm as a result of the subsequent copulatory contact. The resultant conditioning "carried over" to tests 3 and 4 and tended to compensate in part for the lower reactivity that usually obtains during the "day" segment of the diurnal cycle. Unfortunately it was necessary to begin the second series of tests within a week after the first series closed. An appreciably longer interval would probably have decreased the positive carry-over in Group I and produced appreciably lower sex scores for these animals when they were tested in the lighted phase of the illumination cycle.

*Conclusions.* Twenty-seven sexually-inexperienced male rats were maintained under artificial lighting conditions. The living cages were illuminated for 12 hours and darkened for a similar period. All animals were observed in mating tests with receptive females. Males tested during the dark phase of their diurnal cycle were much more active sexually than others tested in the lighted half of the period. When tests were shifted from the light to the dark phase, mating reactions increased promptly. In response to the reverse change sexual activity was reduced. Lowering of responsiveness which accompanied a shift from dark to light phase of the cycle was compensated in part by the effects of experience gained during previous tests conducted in the dark phase. It is concluded that the male rat's tendency to engage in sexual behavior is greater at night than in the day, and that the difference is probably due to the higher metabolic rate which characterizes the early nocturnal portion of the diurnal cycle.

<sup>4</sup> Murlin, J. R., *Ann. Rev. Physiol.*, 1939, 1, 156.

## Circulating Antibodies in Vitamin Deficiency States. Pantothenic Acid Deficiency.\* (17338)

PETER P. LUDOVICI, A. E. AXELROD, AND BETTINA B. CARTER (Introduced by Ralph R. Mellon.)

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In a previous paper, we have reported that pantothenic acid-deficient rats show a severe impairment of antibody response.<sup>1</sup> This finding was in contrast to the observation of Stoerk, Eisen, and John that pantothenic acid deficiency did not affect the level of circulating antibodies.<sup>2</sup> We have attempted to explain this difference in results on the basis that the sheep erythrocytes employed by Stoerk *et al.* were a poorer antigenic stimulus for hemagglutinin production in the rat than the human red blood cells employed by us. Differences in the immunization procedures were also noted. Stoerk, on the other hand, believes that this difference may be attributed to the fact that we were dealing with a relatively more severe pantothenic acid deficiency.<sup>3</sup> Our failure to utilize inanition controls for the pantothenic acid deficient rats, as well as the supposed inadequacy of "paired-fed" controls as contrasted with "paired-weighted" controls were also stressed by this author.<sup>3,4</sup> Some of these points have already been discussed.<sup>5</sup>

The present experiments were planned to determine the effects of varying degrees of pantothenic acid deficiency upon antibody production in the rat utilizing "paired-weighted" animals as inanition controls.

**Experimental.** Fifty-eight male weanling albino rats of the Sprague-Dawley strain were

distributed into 3 groups as indicated in Table I. The animals were housed individually in wide-meshed, screen-bottom cages and weighed daily. Daily food consumption records were taken. All rats were fed a basal diet of the following percentage composition: sucrose, 56.76; Labco "vitamin-free" casein, 25.00; salts,<sup>6</sup> 4.00; cod-liver oil, 2.00; hydrogenated vegetable oil, 10.00; corn oil, 2.00; choline chloride, 0.20; 1-inositol, 0.03; p-aminobenzoic acid, 0.01; and 2-methyl-1, 4-naphthoquinone, 0.001. All rats received additional vitamins in the form of a daily pill. Each of the pills given to the control animals supplied the following vitamins: thiamin, 40  $\gamma$ ; riboflavin, 60  $\gamma$ ; pyridoxine, 50  $\gamma$ ; nicotinic acid, 100  $\gamma$ ; biotin, 1  $\gamma$ ; folic acid, 1  $\gamma$ ; and calcium pantothenate, 300  $\gamma$ . For the pantothenic acid deficient rats, calcium pantothenate was omitted from the pill. The deficient rats were fed the basal diet *ad libitum*. However, each control rat was fed a restricted amount of the basal diet sufficient to maintain its weight equal to that of its pantothenic acid-deficient partner (paired-weighting). After 3, 5, and 7 weeks on experiment, the rats of each group were immunized. A 10% suspension of washed Group O, Rh positive human erythrocytes in physiological saline was injected intraperitoneally as antigen. An initial dosage of 0.5 ml of the red blood cell suspension was followed by 2 ore ml injections on alternate days. Five days after the final injection the rats were bled from the heart under ether anesthesia and the sera tested for agglutinin titers as described previously.<sup>1</sup> Immediately after bleeding, the thymus of each rat was removed and weighed.

**Results.** The individual hemagglutinin titers are recorded in Table II. It is evident that

\* This investigation was supported in part by a grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

1 Axelrod, A. E., Carter, B. B., McCoy, R. H., and Gisinger, R., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 137.

2 Stoerk, H. C., Eisen, H. N., and John, H. M., *J. Exp. Med.*, 1947, **85**, 365.

3 Stoerk, H. C., *Nutrition Rev.*, 1948, **6**, 191.

4 Stoerk, H. C., private communication.

5 Axelrod, A. E., Carter, B. B., and McCoy, R. H., *Nutrition Rev.*, 1948, **6**, 351.

6 Jones, J. H., and Foster, C., *J. Nutrition*, 1942, **24**, 245.



the artificial light cycle is added proof of the greater sexual potency during the "nocturnal" hours.

Female rats tend to come into heat during the night because of the associated rhythm of secretion of gonadal hormones. This rhythm in turn reflects the cyclic secretion of gonadotrophic hormones by the anterior pituitary, the functions of which are influenced by periodic increase and decrease in the amount of environmental illumination as well as concomitant changes in external temperature. Regular fluctuations in hypophyseal activity also affect the secretory behavior of the thyroid and probably of still other endocrine glands. As a result, the albino rat displays a diurnal rhythm of energy metabolism which begins to increase at approximately noon, reaches a maximum around 9 P.M. and progressively declines to the next noon. If rats are placed under constant illumination and fed at frequent intervals, the metabolic rhythm is abolished.<sup>4</sup>

There is no reason to believe that the rat testis secretes androgen at significantly different rates during different times of day, and even if it did so the behavioral response to alteration in the concentration of testicular hormone is too slow to account for the diurnal variations that characterize the male's mating performance. The heightened sexual excitability shown by male rats during the dark phase of the illumination cycle probably is due to the nocturnal increase in basal metabolism and to a consequent increase in responsiveness to all kinds of exteroceptive stimulation.

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when these animals were mated during their "night." In tests 1 and 2 the majority of Group I males gained a considerable amount of sexual experience. They became accustomed to receiving receptive females in the observation cage and achieved orgasm as a result of the subsequent copulatory contact. The resultant conditioning "carried over" to tests 3 and 4 and tended to compensate in part for the lower reactivity that usually obtains during the "day" segment of the diurnal cycle. Unfortunately it was necessary to begin the second series of tests within a week after the first series closed. An appreciably longer interval would probably have decreased the positive carry-over in Group I and produced appreciably lower sex scores for these animals when they were tested in the lighted phase of the illumination cycle.

*Conclusions.* Twenty-seven sexually-inexperienced male rats were maintained under artificial lighting conditions. The living cages were illuminated for 12 hours and darkened for a similar period. All animals were observed in mating tests with receptive females. Males tested during the dark phase of their diurnal cycle were much more active sexually than others tested in the lighted half of the period. When tests were shifted from the light to the dark phase, mating reactions increased promptly. In response to the reverse change sexual activity was reduced. Lowering of responsiveness which accompanied a shift from dark to light phase of the cycle was compensated in part by the effects of experience gained during previous tests conducted in the dark phase. It is concluded that the male rat's tendency to engage in sexual behavior is greater at night than in the day, and that the difference is probably due to the higher metabolic rate which characterizes the early nocturnal portion of the diurnal cycle.

<sup>4</sup> Murlin, J. R., *Ann. Rev. Physiol.*, 1939, **1**, 156.

the antibody response was decreased in the pantothenic acid-deficient rats of all groups. In no instance, did the titer of a deficient rat equal that of its "paired-weighed" control. The deleterious effect of a mild pantothenic acid deficiency is evidenced by the decreased titers of the animals in Group I which were on experiment for only 3 weeks prior to immunization. This observation lends no support to the explanation offered by Stoerk for the discrepancy in the findings of the two laboratories. The comparatively high antibody content of the "paired-weighed" controls is further evidence against the role of inanition in the decreased antibody response of pantothenic acid-deficient rats.

In the course of further studies on the mechanism of action of pantothenic acid we have determined the antibody response in 44 pantothenic acid-deficient rats and 29 suitable controls. The results obtained are in excellent

agreement with those recorded in this as well as in a previous paper.<sup>1</sup> All of the evidence to date firmly implicates pantothenic acid as a vital factor in the antibody response to the antigenic stimulus of human erythrocytes.

The thymus weights of the pantothenic acid-deficient rats were lower than those of the control group (Table I). These findings are in agreement with those made by Stoerk *et al.*<sup>2</sup>

*Summary.* (1) Hemagglutinin production in response to inoculation with human erythrocytes has been investigated in rats fed a pantothenic acid-deficient diet for 3, 5, and 7 weeks. "Paired-weighed" animals served as inanition controls.

(2) Marked impairment of antibody response was observed in all pantothenic acid-deficient groups.

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## Selective XYZ Factor in C57 Black Mammary Carcinoma Eo771. (17339)

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The XYZ phenomenon may be defined as the increased incidence and more rapid increment of local and metastatic transplanted tumors following the prior injection in the same or distant sites of an extract from the homologous tumor.<sup>1-4</sup> The factor in the

Brown-Pearce rabbit tumor responsible for the phenomenon is selective, filtrable (Berkefeld "V") and thermolabile (56°C);<sup>5-7</sup> that in Bashford mouse mammary carcinoma 638.<sup>8</sup> is likewise highly selective or specific.<sup>10-12</sup>

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TABLE I.  
Summary of Growth, Food Consumption, and Thymus Weight Data.\*

Type	No. of rats	Body wt†		Thymus‡ wt	Daily food consumption‡
		Initial	Final§		
Group I*					
Control	10	42	83	195	4.4
Pantothenic acid-deficient	10	42	84	116	4.8
Group II*					
Control	10	43	83	140	4.0
Pantothenic acid-deficient	10	43	84	78	4.4
Group III*					
Control	9	42	94	90	3.9
Pantothenic acid-deficient	9	43	96	69	4.7

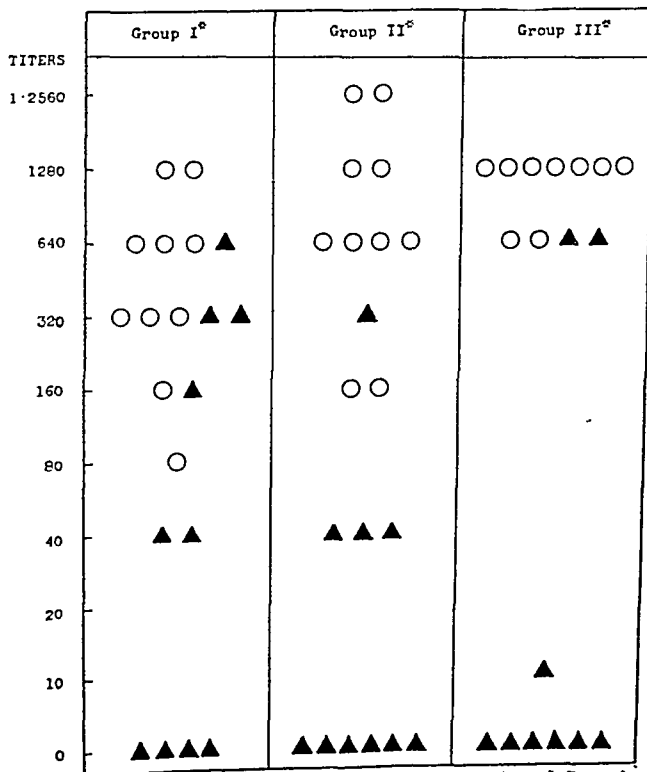
\* Groups I, II, III were fed basal diet 3, 5, and 7 weeks respectively prior to immunization

† Group average in g.

‡ Group average in mg.

§ At the time of bleeding.

Table II.  
Individual Hemagglutinin Titers.



\* Groups I, II, III were fed the basal diet 3, 5, and 7 weeks, respectively, prior to immunization.

○—Control animals.

▲—Pantothenic acid-deficient animals.

TABLE I.

Experiments with XYZ Factor from Mammary Carcinoma Eo771, Using C57 Black Mice.

Injection of XYZ factor				Data on tumor transplantation and growth						
Origin of XYZ	Stored		Days prior	Tumor and its origin	Host mice	Total mice	Grew tumor	$\Sigma x$	$M_x$	$\Sigma x_2$
	Days	Temp.								
A. Heterologous XYZ and Heterologous Host.										
Eo771 57bl	45	0°F	34	15091a A	57bl	10	0	0.00	0.00	0.0
Controls				15091a A	57bl	9	0	0.00	0.00	0.0
B. Heterologous XYZ and Homologous Host.										
Ba. Ca. C3H	337	0°F	16	Eo771 57bl	57bl	10	9	23.70	2.37	108.5
Controls						10	10	26.90	2.69	102.2
Eo771 57bl	45	0°F	43	241-5 57bl	57bl	10	5			
Eo771 57bl	45	0°F	43	241-5 57bl	57bl	8	2	15.42	0.86	56.5
Controls				241-5 57bl	57bl	8	6			
Controls				241-5 57bl	57bl	10	3	57.31	3.18	469.0
Eo771 57bl	45	0°F	50	241-16 57bl	57bl	8	4	10.14	1.27	85.0
Controls				241-16 57bl	57bl	20	12	36.52	1.83	216.8
241-5 57bl	68	0°F	10	Eo771 57bl	57bl	9	7			
241-5 57bl	68	0°F	10	Eo771 57bl	57bl	9	8	65.36	4.36	450.3
Controls				Eo771 57bl	57bl	10	6			
Controls				Eo771 57bl	57bl	10	10	74.64	3.73	459.5
241-5 57bl	68	0°F	34	Eo771 57bl	57bl	9	5	10.38	1.15	23.8
Controls				Eo771 57bl	57bl	9	8	17.18	1.91	58.4
Summary XYZ injected animals						63	40	124.94	1.98	723.3
Summary controls						77	55	212.65	2.76	892.0
C. Homologous XYZ and Homologous Host.										
Eo771 57bl	45	0°F	25	Eo771 57bl	57bl	9	9			
Eo771 57bl	45	0°F	25	Eo771 57bl	57bl	10	10	135.37	7.12	1352.0
Controls				Eo771 57bl	57bl	10	6			
Controls				Eo771 57bl	57bl	8	8	52.86	2.94	302.4
Eo771 57bl	28	0°F	18	Eo771 57bl	57bl	10	10	63.44	6.34	472.5
Eo771 57bl	28	0°F	19	Eo771 57bl	57bl	10	9	4.98	0.50	3.9
Controls				Eo771 57bl	57bl	9	7	4.25	0.47	3.5
Controls				Eo771 57bl	57bl	8	8	25.72	3.22	110.1
Summary XYZ injected animals						39	38	203.79	5.23	1828
Summary controls						35	29	82.83	2.37	416

urement in each of the 12 experiments, the measurements used were the last series taken before the first animals of either test or control groups died from the tumor. The tumors employed were Eo771, 241-5, and 241-16, mouse mammary carcinomata, of C57 black origin; Barrett mammary carcinoma of C3H origin; and 15091a mammary carcinoma of Line A origin.<sup>†</sup> In each experiment Eo771 was used either as the source of the XYZ material or as the source of the tumor tissue, or both.

<sup>†</sup> The mouse tumors were obtained through the courtesy of various persons: Eo771 and 15091a from Dr. George D. Snell of Bar Harbor, Maine, 241-5 and 241-16 from Dr. Howard B. Andervont, Dr. Thelma Dunn and Dr. Harold L. Stewart, and the Barrett carcinoma from Dr. M. K. Barrett, and C57 black mammary carcinoma 755 from Dr. Edwin D. Murphy, all of Bethesda, Md.

Since Eo771, 241-5 and 241-16 originated in C57 black mice, such mice were considered "homologous hosts" when any of the 3 tumors was transplanted into them. On the other hand 15091a (of Line A origin) transplanted in C57 black mice, was said to have been inoculated into a heterologous host.

The XYZ material, if from a different tumor than that transplanted, was said to be heterologous, even though originating in the same anatomic site in the same inbred strain (*i.e.* 241-5, 241-16 and Eo771 in C57 black mice). Experiments with another mammary carcinoma No. 755 originating also in C57 black mice<sup>19</sup> are now in progress.

The XYZ material was prepared by placing fresh aseptically removed tumor tissue in

<sup>19</sup> Kaplan, Henry S., and Murphy, Edwin D., *J. Nat. Cancer Inst.*, 1949, 9, 407.

Brown-Pearce and Bashford tumor tissues aseptically removed from the host, frozen immediately and kept frozen at 0-24°F until the cells are no longer viable uniformly contain the respective XYZ factors in great quantity; cultures for bacteria have been sterile<sup>8,9,13</sup> and the Brown-Pearce tumor tissue has been free from Virus III, rabbit pox and other known viruses.<sup>14</sup> No reaction appears at the site of single or repeated injections of the XYZ materials. Similarly prepared extracts of normal tissues or of other tumors even in the same species when injected prior to transplantation of Bashford 63 or the Brown-Pearce tumor have not elicited the XYZ phenomenon.<sup>5,13,15</sup>

Recently Snell, Cloudman, Failor, and Douglass,<sup>16</sup> and Snell, Cloudman, and Woodworth<sup>17</sup> have reported similarly planned experiments using inbred strains of mice. Their data indicate to us enhancement of homologous tumor growth entirely acceptable to the above definition of the XYZ phenomenon by factors present in C57 Black mouse mammary carcinoma Eo771 and Line A mouse mammary carcinoma 15091a. Their technic consisted in freezing and storing the tumor tissue with lyophilization as an added routine procedure. Lyophilization has been found to be an effective method of storing the XYZ factor from the Brown-Pearce tumor.<sup>18</sup> Desiccation after freezing of the Brown-Pearce tumor by the methods current in 1930, resulted in some loss in the potency of the XYZ factor,<sup>1</sup> and the more modern lyophilization methods such as those used by Snell, Cloud-

man, and Woodworth are a great improvement in the study of the XYZ factors.

The present communication reports experiments by us with Eo771 and correlates our results with those previously obtained by Snell, Cloudman, and Woodworth.

*Material and methods.* The animals were 250 C57 black mice received from Carworth Farms, New City, New York over a period of one year in batches of 40 (animals of each batch of same sex). On receipt the animals were placed in metal self-feeder mouse boxes 10 to a box, and alternate boxes marked "control" and "experimental" respectively. The animals were allowed from 1-4 months to mature and become adjusted to the new environment. The animal quarters were air conditioned throughout the year, temperature set at 70°F and humidity at "ideal." Some 8 experimental and 9 control mice died of intercurrent disease over an average 6-8 months observation period (loss 6.8%) and usually before experiments were initiated. Since the diagnosis in each was checked by necropsy and histologic examination these 15 mice were eliminated from the series. In no instance did injection of XYZ material lead to local infection or abscess. Except in 2 experiments (40 mice) no evidence of infection appeared at the sites of tumor transplantation. In the 2 experiments above mentioned abscesses (4-5 mm in diameter) appeared at the site of tumor transplantation in about 30-40% of control as well as experimental animals. In each instance transfer had been made from a large growth having considerable necrosis. The number of takes in the 2 experiments was less than normal, although good tumors eventually grew from most of the animals. No animal died because of the intradermal abscesses, and all lesions were healed for the most part by 15 days.

There were 12 separate experiments with equal numbers in control and experimental series with one exception in which there were 20 controls instead of the usual 10 (Table I). Measurements of the tumors in 3 dimensions in the controls and experimental animals were made concomitantly at intervals after inoculation of the tumor tissue. Although the trends remained uniform from the first meas-

<sup>13</sup> Drysdale, George R., and Casey, Albert E., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 306.

<sup>14</sup> Pearce, Louise, and Casey, Albert E., 1933 (unpublished experiments, The Rockefeller Institute for Medical Research).

<sup>15</sup> Casey, Albert E., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 731.

<sup>16</sup> Snell, George D., Cloudman, Arthur M., Failor, E., and Douglass, P., *J. Nat. Cancer Inst.*, 1946, **6**, 303.

<sup>17</sup> Snell, George D., Cloudman, Arthur M., Woodworth, Eliz., *Cancer Research*, 1948, **8**, 429.

<sup>18</sup> Drysdale, George R., Ross, Gordon L., Skipper, Howard, Edwards, P. C., and Casey, Albert E., unpublished experiments.

TABLE II.

Other Experiments with C57 Black Mouse Mammary Carcinoma Eo771. (Data abstracted and rearranged from the paper by Snell, Cloudman, and Woodworth<sup>17</sup> to correspond with the format in Table I).

Injection of XYZ factor			Data on tumor transplantation and growth					
Origin of XYZ	Stored		Days prior	Tumor and its origin	Host mice	Total mice	Died, tumor	Maximum mean tumor size, sqcm
	Days	Temp.						
A. Heterologous XYZ and heterologous host.								
Eo771 57bl	?	lyoph.	8-12	C1498 57bl	57ln	6	4	5.53
Eo771 57bl	?	"	8-12	C1498 57bl	57ln	10	3	0.30
L946 57bl	?	"	8-12	Eo771 57bl	57ln	10	10	9.82
Controls				C1498 57bl	57ln	10	0	0.65
"				C1498 57bl	57ln	10	6	1.94
"				C1498 57bl	57ln	10	7	?
"				C1498 57bl	57ln	10	7	?
"				Eo771 57bl	57ln	8	8	12.99
Summary XYZ injected animals						26	17	5.17
Summary controls						48	28	4.64
B. Heterologous XYZ and homologous host (no experiments reported).								
C. Homologous XYZ and homologous host (no experiments reported).								
D. Homologous XYZ and heterologous host.								
Eo771 57bl	?	lyoph.	8-12	Eo771 57bl	57ln	10	8	3.26
Eo771 57bl	?	"	8-12	Eo771 57bl	57bred	10	0	0.25
Eo771 57bl	?	"	15	Eo771 57bl	C58	15	15	4.97
Eo771 57bl	?	"	8-12	Eo771 57bl	C58	10	7	2.31
Eo771 57bl	?	"	8-12	Eo771 57bl	BAlbC	12	3	0.17
Eo771 57bl	?	"	8-12	Eo771 57bl	BAlbC	12	8	5.94
Eo771 57bl	?	"	8-12	Eo771 57bl	BAlbC	9	7	3.90
Controls				Eo771 57bl	57ln	5	3	4.25
"				Eo771 57bl	57bred	10	0	0.13
"				Eo771 57bl	C58	14	2	0.24
"				Eo771 57bl	C58	10	0	0.13
"				Eo771 57bl	BAlbC	12	0	0.42
"				Eo771 57bl	BAlbC	10	0	0.44
Summary XYZ injected animals						78	48	3.09
Summary controls						61	5	0.60

larging tumors among 48 controls (58%). This difference in incidence was not significant ( $X^2 = 0.356$ ;  $n = 1$ ;  $P = 0.6$ , not significant). The average size of the tumors in the 26 heterologous XYZ injected mice was 5.17 sq cm as compared with 4.64 sq cm among the 48 controls. The difference  $0.53 \pm 0.47$  sq cm was not statistically significant.

When the data for heterologous XYZ injected animals of Tables I and II with their respective controls were combined there were 57 progressively enlarging tumors among 99 heterologous XYZ animals (58%), and 83 progressively enlarging tumors among 134 controls (62%). The difference was not statistically significant. The mean size of the tumors in the two groups was almost identical and the data of Snell, Cloudman and Woodworth and ours are in entire agreement. To us the data indicates that heterologous XYZ material has no appreciable effect on

transplanted tumor growth in either homologous or heterologous hosts.

In 7 experiments 48 of the 78 mice injected with homologous XYZ material by Snell, Cloudman, and Woodworth had progressively enlarging tumors (62%) as compared with 5 tumors among 61 control mice (8%). This difference was statistically significant ( $X^2 = 40.4$ ;  $n = 1$ ;  $P = 0.0001$ —significant). The tumors among the 78 experimental mice averaged 3.09 sq cm and among 61 controls 0.60 sq cm. The difference  $2.49 \pm 0.34$  sq cm was statistically significant ( $t = 4.3$ ;  $P = 0.0001$ —significant). The key to the puzzle would seem to be the use of homologous XYZ material, and with such material heterologous inbred hosts were not always resistant to tumors of foreign strain origin.

In the experiments summarized in Tables I and II cross XYZ reactions were studied among 5 neoplasms of C57 black origin, and

the deep freeze chamber at 0°F and keeping it there for 28-337 days. In each instance a portion of the tumor tissue to be frozen was tested for viability by routine transfer. Each tumor for XYZ or for transplant was verified by histologic examination, using the blind check method for tumor identification. 241-5 and 241-16 were quite similar histologically and cage records were necessary to differentiate between them.

Since the Brown-Pearce and Bashford 63 XYZ factors withstand storage in the cold for at least a year and since a single injection leaves prolonged hypersusceptibility to the same tumor, some latitude was exercised in the storage (45-337 days) and interval before reinoculation (10-50 days) in the Eo771 experiments (Table I). The measurements of the transplanted tumor were made on the same day for both control and test mice, using calipers. The "volume" was the product of the caliper diameters in 3 dimensions. Although this introduced a considerable error when checked by weight of the tumors it gave a uniform value for both test and control animals.

The XYZ material and the tumor tissue for transplantation were prepared for inoculation by mincing and grinding in a mortar and diluting with normal saline (1 part tumor and 3 parts saline). In the last 2 experiments (Table I) Penicillin G sodium (30,000 units cc) buffered with sodium citrate (Lederle) was used as the diluent in the place of normal saline. 0.1 to 0.15 cc of the emulsion of the frozen XYZ or of the fresh tumor tissue as the case might be, was inoculated subcutaneously into the left groin.

A necropsy with histologic examination of lungs, heart, liver, and primary inoculation site and other selected tissue was made on each of the 250 mice. Because of the rapid growth of the tumors experiments in which the animals were transplanted with Eo771 were terminated at 15-30 days, whereas experiments with 241-5 and 241-16 were not terminated before 40-70 days depending on when the first animal died.

**Results.** The results are presented in tabular form under 3 categories, namely: A. Heterologous XYZ and heterologous host; B. Hetero-

logous XYZ and homologous host; C. Homologous XYZ and homologous host (Table I). In no instance did a heterologous XYZ material produce an XYZ effect in either a heterologous or in a homologous host (A and B, Table I). The summarized material indicates that 40 of the 63 heterologous XYZ injected animals (63%) had progressively enlarging tumor growths with an average size of 1.98 cc whereas 55 of the 77 control mice (71%) had progressively enlarging growths which averaged 2.76 cc (the difference  $\approx 0.78 \pm 0.42$  cc;  $t = 1.9$ ;  $n = 138$ ;  $P = 0.06$ , not significant). This difference would indicate that the heterologous XYZ material produced a statistically insignificant inhibition of tumor growth. Likewise there was an insignificant inhibition of the incidence of transplanted tumors among the XYZ animals ( $X^2 = 0.99$ ;  $n = 1$ ;  $P = 0.4$ , not significant). The mean tumor size when the data for the Barrett carcinoma experiment was eliminated was 2.77 cc for 67 control mice as compared with 1.91 cc for 53 heterologous XYZ injected mice. The difference  $0.86 \pm 0.45$  cc is suggestive of an inhibition.

In contrast to the above data the 39 animals injected with homologous XYZ into an homologous host had 38 progressively enlarging primary tumors (97%) as compared, with 29 progressively enlarging tumors among the 35 control mice in the 4 experiments (83%). The tumors averaged among the 39 XYZ injected animals 5.23 cc as compared with 2.37 cc among the 35 controls. The difference  $2.86 \pm 0.84$  cc is statistically significant ( $t = 3.4$ ;  $n = 72$ ;  $P = 0.01$ —significant).

**Discussion.** In order to provide a basis for comparison with present results, the data of Snell, Cloudman, and Woodworth<sup>17</sup> pertaining to Eo771 has been abstracted and rearranged in the manner of the present experiments (Table II). This data includes observations on 213 inbred mice in which either Eo771 XYZ or Eo771 tumor transplants or both were used. There were three experiments with heterologous XYZ in heterologous hosts. The 26 XYZ injected animals had 17 progressively enlarging primary tumors (65%) as compared with 28 progressively en-

ed hosts, so long as the tumor cells were viable in the foreign host.

3. XYZ factors have thus far been proven for only 4 transplanted neoplasms of mice and rabbits all of which are Grade III-IV by his-

tologic grading. No such factors have been observed in adult tissues, leukemias, benign and low grade tumors.

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### *In vitro* Determination of Bacterial Sensitivity to Aureomycin. (17340)

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Results of aureomycin sensitivity studies of various microorganisms have deviated greatly from one another as reported by different laboratories. This has been best exemplified by wide divergences in the reported sensitivities of staphylococci to this agent. Paine and associates<sup>1</sup> employing both tube dilution and plate methods, noted that 22 strains of *S. aureus* required 1 to 2  $\mu$ g of aureomycin per ml for inhibition. The 5 remaining strains were inhibited by higher concentrations of the antibiotic, ranging up to 12.5  $\mu$ g per ml. The type of media used was not described. Price and collaborators<sup>2</sup> reported *S. aureus* to be inhibited by a range of .09 to 25  $\mu$ g of aureomycin per ml. The determinations were made following incubation of the organisms in penicillin assay broth for 24 hours. Bryer and associates<sup>3</sup> stated that 0.6  $\mu$ g of aureomycin per ml inhibited staphylococci. Their report contained no statement about the method of determination. Twenty-one strains of this group of organisms tested by Lankford and Lacy<sup>4</sup> were inhibited by .05 to 0.2  $\mu$ g of aureomycin per ml. Bacto-tryptose agar was the medium employed. With a tryptose broth turbidimetric method and 50% inhibition of growth in 18 hours as the end-point, the most

sensitive strain was inhibited by .012  $\mu$ g of aureomycin per ml and the most resistant by .033  $\mu$ g per ml.

This study is an investigation of possible causes for discrepancies in the results obtained by others. In addition, a search has been made for a simple and reliable method of determining bacterial sensitivity to aureomycin.

**Methods.** Growth curves of *S. aureus*, enterococcus, *S. panama*, and *E. coli* were determined by a turbidimetric method. 0.1 ml inocula of 18 hour broth cultures were added to 18 mm test tubes containing 10 ml of tryptose broth (Difco). Aureomycin crystals were dissolved in cold sterile distilled water, diluted in tryptose broth, and added to the system immediately.\* The final concentrations were .01, .02, .05, 0.1, 0.2, 0.5, 1, 2, 5, and 10  $\mu$ g per ml. A control tube without aureomycin was included in each determination. The cultures were incubated at 37°C and the turbidity was measured in a Coleman Junior spectrophotometer with a 650 m filter. Values of less than 0.1 unit of optical density indicated marked bacteriostasis and were chosen as end-points.

Tryptone glucose extract agar with para-aminobenzoic acid (Difco) was the medium used for the plate method. Aureomycin was incorporated in this medium in final concentrations identical with those described above plus 20 and 50  $\mu$ g per ml, and 18 hour broth cultures were streaked on the plates. Readings were made at various intervals of time,

\* Solutions of aureomycin in water retained full potency if stored in the frozen state.

<sup>1</sup> Paine, T. F., Collins, H. S., and Finland, M., *J. Bact.*, 1948, 56, 489.

<sup>2</sup> Price, C. W., Randall, W. A., and Welch, H., *Ann. New York Acad. Sci.*, 1948, 51, 211.

<sup>3</sup> Bryer, M. S., Schoenbach, E. B., Chandler, C. A., Bliss, E. A., and Long, P. H., *J.A.M.A.*, 1948, 138, 117.

<sup>4</sup> Lankford, C. E., and Lacy, H., *Texas Rep. Biol. Med.*, 1949, 7, 111.



the reactions in a sixth are now being tested (mammary carcinoma 755). Four of the 6 are mammary carcinomata of C57 black origin. (15091 of Line A origin, and Barrett carcinoma of C3H origin are also mammary carcinomata). If the XYZ material be of cellular origin it must be a permanent chemical mutation not present in the other mammary tissues and tumors of the same inbred strain. It must be perpetuated ad infinitum in the specific cells, but in no other and must exert a powerful influence on the continued transplantability and growth of the specific cells.

If the XYZ material be an inert contaminating virus<sup>20</sup> (a possibility which was considered early in the work)<sup>14</sup> it might conceivably act as follows: The injected XYZ material immunizes the host to the virus and the subsequently transplanted tumor grows with untrammelled or natural vigor, unimpeded by its contaminating virus.<sup>21,25</sup> This possibility was considered with the Brown-Pearce rabbit tumor XYZ material. A Lilac rabbit (resistant strain), in which a Brown-Pearce transplant had grown subcutaneously, regressed and disappeared, was sacrificed. Blood serum from this "immune" rabbit was mixed in equal parts with XYZ material and allowed to stand for some hours in a kitchen type ice box. This immune serum-XYZ mixture injected prior to tumor transplantation enhanced the XYZ effect. If an inert contaminating virus were present, then, the virus-serum mixture perhaps should have lessened the XYZ effect instead of increasing it. Also probably every strain of Brown-Pearce tumor throughout the world today was derived from animals which had had XYZ material before tumor inoculation and shipped (by AEC) to various places. Our own strains came from several generations of such XYZ animals yet the XYZ material may be obtained with regularity and ease from the frozen tissue. If a virus, it is inert in the sense that no fatalities or clinical

disease follows its injection, and dynamic in the sense that metastases and local growth of the homologous tumor are enhanced by its injection. The recent experimental work of Kaplan and Murphy on insufficient radiation inducing more metastases is very suggestive of the XYZ effect.<sup>19</sup> The Brown-Pearce material is almost equally as effective when given 2 weeks after tumor transplantation as 2 weeks before.<sup>1</sup> The XYZ effect does not result when Berkefeld or Seitz filtrates of fresh tumor<sup>22,23</sup> or nuclei and cytoplasm of fresh tumor<sup>24</sup> are injected prior to tumor transplantation. A single injection of the XYZ factor into immune rabbits which had been tested and retested by inoculations of Brown-Pearce tumor broke down the resistance of 33% of the animals upon subsequent challenge with fresh tumor tissue.<sup>1</sup>

*Summary and conclusions.* 1. Twelve experiments were made with mouse mammary carcinoma Eo771 (C57 black origin) using 250 C57 black mice. Complementary use was made of mouse mammary carcinomata 241-5, 241-16 (C57 black origin), 15091a (Line A origin), and Barrett (C3H origin).

2. A highly selective XYZ factor (entirely comparable with those described for the Brown-Pearce rabbit tumor and Bashford mouse mammary carcinoma 63), resulting in enhanced tumor growth, was found to be uniformly present in the frozen Eo771 tumor tissue. The XYZ effect was present when the XYZ factor was derived from the homologous tumor and absent when derived from a heterologous tumor, even from other mammary carcinomata of the same highly inbred C57 black strain. This high tissue selectivity of the XYZ factor was not limited or determined by the host into which the tumor cells were transplanted. The factor was equally effective in homologous or heterologous transplant-

<sup>20</sup> Taylor, M. J., and MacDowell, E. C., *Cancer Research*, 1949, 9, 144.

<sup>21</sup> Kritzer, Robert A., Mulliken, B., and Turner, Joseph C., *Cancer Research*, 1949, 9, 74.

<sup>22</sup> Casey, Albert E., *Proc. Soc. Exp. Biol. and Med.*, 1936, 34, 111.

<sup>23</sup> Drysdale, George R., and Casey, Albert E., unpublished experiments.

<sup>24</sup> Skipper, Howard, Edwards, P. C., Drysdale, George R., and Casey, Albert E., unpublished experiments.

<sup>25</sup> Bang, F. B., and Gey, G. O., *Proc. Soc. Exp. Biol. and Med.*, 1949, 71, 78.

PLATE II.  
Plate Method.

Hours	Tryptone glucose extract agar-PABA						Tryptone glucose extract agar-PABA Serum						Tryptone glucose extract agar-PABA Blood					
	12	18	24	36	60		12	18	24	36	60		12	18	24	36	60	
<i>S. aureus</i> (41552)	.05-.2	1-.5	.5	.5	1-20		.5-1	1-2	2	5-10	10-50		.5-1	1-2	2	5	20	
<i>S. aureus</i> (42044)	.05-1	1-.5	2-.5	.5-1	1-2		.5-1	1-2	2	5	5-20		.5-1	2	5	5	20	
Enterococcus (44962)	1-.5	.5	.5-1	1-2	10-20		.5-1	1-2	2	5-10	10-over 50		.5-1	1-2	2-5	10	20-50	
<i>Sal. panama</i> (40615)	1-2	1-2	2-5	5-10	10-20		2-5	2-10	10	5-20	10-over 50		5-10	10	10-20	20-50	50-over 50	
<i>E. coli</i> (41040)	1-2	2	5	5-20	5-50		2	2-10	10	5-over 50	10-over 50		2-5	5	10	50	Over 50	
<i>S. aureus</i> (37215)	.05-.2	1-2	1-2		1-2													
Enterococcus (40056)	1-2	2-1		5-1	1-2								.5-1	1-2	2-5	10	20-50	

The figures represent the concentrations of aureomycin in  $\mu\text{g}$  per ml, required to inhibit growth.

the magnitude described above as inhibitory were present. No lysis was evident even with concentrations of the agent one hundred times greater than those sufficient for bacteriostasis.

The common ranges of aureomycin sensitivity determined by the plate method for 6 strains of *S. aureus* were .05 to 0.2  $\mu\text{g}$  per ml at 12 hours, 0.1 to 0.5  $\mu\text{g}$  per ml at 18 hours, 0.5 to 1  $\mu\text{g}$  per ml at 24 hours, 0.5 to 5  $\mu\text{g}$  per ml at 36 hours, and 1 to 20  $\mu\text{g}$  per ml at 60 hours (See Tables II and III). The values for 5 strains of enterococcus were 0.1 to 0.5  $\mu\text{g}$  per ml at 12 hours, 0.2 to 1  $\mu\text{g}$  per ml at 18 hours, 0.5 to 1  $\mu\text{g}$  per ml at 24 hours, 0.5 to 2  $\mu\text{g}$  per ml at 36 hours, and 1 to 20  $\mu\text{g}$  per ml at 60 hours. Aureomycin inhibitory levels for *Sal. panama* were 1 to 2  $\mu\text{g}$  per ml at 12 and 18 hours, 2 to 5  $\mu\text{g}$  per ml at 24 hours, 5 to 10  $\mu\text{g}$  per ml at 36 hours, and 10 to 20  $\mu\text{g}$  per ml at 60 hours. The *E. coli* values were about the same as those for *Sal. panama*. Considerably higher levels were required for inhibition when serum or blood was present. At 18 hours, for example, these ranged from 2 to 20 times greater for the gram positive cocci, and 1 to 10 times increase in aureomycin concentration for the gram negative bacilli.

Four strains of *S. aureus* had been tested against penicillin and streptomycin. Three were penicillin-resistant and one was streptomycin-resistant. All strains were equally sensitive to aureomycin.

**Discussion.** The data presented in this study are in close agreement with those of Lankford and Lacy.<sup>4</sup> Very low concentrations of aureomycin inhibited *S. aureus* if the medium used was plain tryptone broth or tryptone glucose extract agar. The same was true of other organisms tested. Lowest values were obtained with the 4 hour turbidimetric method. Addition of serum or blood to the medium increased the concentration of aureomycin required for inhibition, especially when incubation was prolonged. This effect was quite marked with the plate method. The absence of bactericidal action by aureomycin and the rapid deterioration of this agent during incubation offer an adequate explanation of these observations. A richer medium would be expected to accelerate bacterial growth

TABLE I.  
Turbidimetric Method. (Concentration of aureomycin in  $\mu\text{g}$  per ml).

	Tryptose broth—hr						Tryptose broth plus 10% serum—hr					
	4	14	20	40	50	60	4	14	20	40	50	60
<i>S. aureus</i> (41552)	.02-.05	.1-.2	.2-.5	.5	—	1-5	.05	.2-.5	.5	—	—	5
<i>S. aureus</i> (37215)	.05	2	—	—	.5	—	.05	.5	—	—	5	—
<i>Enterococcus</i> (40056)	.1	—	.5	—	5	—	.2	—	2	—	10	—
<i>Sal. panama</i> (40615)	.5	1-2	2-5	10	—	—	.5	2	5	10 or more	—	—
<i>E. coli</i> (41010)	.5	1	2	—	10	—	.5-1	1	5	—	—	—

The figures represent the concentration of aureomycin in  $\mu\text{g}$  per ml, required to inhibit growth.

complete inhibition of growth being the end-point.

A number of sensitivity determinations were made with pooled sterile human sera or whole horse blood added to the basal media. Two percent hemolyzed horse blood was used for the turbidimetric method. The blood added to tryptone glucose extract agar invariably hemolyzed.

The one precaution to be consistently observed is rapid preparation and inoculation of the aureomycin media. The whole task should be performed within a few hours due to rapid deterioration of this antibiotic.

**Results.** Inhibitory levels of aureomycin for 2 strains of *S. aureus* as determined by the turbidimetric method were .02 to .05  $\mu\text{g}$  per ml after 4 hours of incubation, 0.1 to 0.2  $\mu\text{g}$  per ml after 14 hours, 0.2 to 0.5  $\mu\text{g}$  per ml after 20 hours, 0.5  $\mu\text{g}$  per ml in 40 to 50 hours, and 1 to 5  $\mu\text{g}$  per ml after 60 hours (See Table I). A composite growth curve derived from 9 experiments is presented in Fig. 1. It demonstrates partial inhibition for *S. aureus* by .01 and .02  $\mu\text{g}$  of aureomycin per ml, complete inhibition for 7 hours by .05  $\mu\text{g}$  per ml, and failure of this and higher concentrations to inhibit bacterial growth as the period of incubation is lengthened. One strain of enterococcus was inhibited by 0.1  $\mu\text{g}$  per ml at 4 hours, 0.5  $\mu\text{g}$  per ml at 20 hours, and 5  $\mu\text{g}$  per ml at 50 hours. *Salmonella panama* was inhibited by 0.5  $\mu\text{g}$  per ml at 4 hours, 1 to 2  $\mu\text{g}$  per ml at 14 hours, 2 to 5  $\mu\text{g}$  per ml at 20 hours, and 10  $\mu\text{g}$  per ml at 40 hours. For one strain of *E. coli*, the inhibitory levels were 0.5  $\mu\text{g}$  per ml at 4 hours, 1  $\mu\text{g}$  per ml at 14 hours, 2  $\mu\text{g}$  per ml at 20 hours, and 10  $\mu\text{g}$  per ml at 50

hours. With serum present in 10% concentration, the levels were the same or somewhat higher. Increases were usually to the extent of one tube dilution, but were occasionally 5 to 10-fold (3 to 4 tube dilutions) following long periods of incubation.

Subculture of the media in the tubes containing 10  $\mu\text{g}$  of aureomycin per ml invariably revealed a few viable organisms. *S. aureus* so isolated was inhibited by .02  $\mu\text{g}$  of aureomycin per ml at 4 hours and 0.1  $\mu\text{g}$  per ml at 20 hours. For *Sal. panama*, the values were 0.5  $\mu\text{g}$  per ml at 4 hours and 2  $\mu\text{g}$  per ml at 14 hours. These values were comparable to those obtained with the parent strains.

Aureomycin incubated for 18 hours at 37°C was tested against *S. aureus* and *Sal. panama*. The inhibitory levels for *S. aureus* were 0.2  $\mu\text{g}$  per ml at 4 hours and 1  $\mu\text{g}$  per ml at 20 hours. Pre-incubation levels were .02  $\mu\text{g}$  per ml and 0.2  $\mu\text{g}$  per ml at 4 and 20 hours respectively. Two micrograms per ml at 4 hours and 5 to 10  $\mu\text{g}$  per ml at 20 hours were required by *Sal. panama* as compared with control levels of 0.5  $\mu\text{g}$  per ml at 4 hours and 2  $\mu\text{g}$  per ml at 20 hours. This represents about 60 to 90% deterioration of aureomycin in 18 hours.

The aureomycin inhibitory levels observed following incubation of *S. aureus* and *Sal. panama* for 4 hours in tryptose broth containing 2% horse blood were .05  $\mu\text{g}$  and 0.5  $\mu\text{g}$  per ml respectively. These values corresponded closely to those obtained with the simple tryptose broth medium.

Aureomycin was added to cultures of *S. aureus*, *E. coli*, and enterococcus one or 2 hours after growth commenced. Further growth was inhibited if aureomycin levels of

PLATE II.  
Plate Method.

Hours	Tryptone glucose extract agar-PABA					Tryptone glucose extract agar-PABA Serum					Tryptone glucose extract agar-PABA Blood				
	12	18	24	36	60	12	18	24	36	60	12	18	24	36	60
<i>S. aureus</i> (41552)	.05-2	1-5	.5	.5	1-20	.5-1	1-2	2	5-10	10-50	.5-1	1-2	2	5	20
<i>S. aureus</i> (42044)	.05-1	1-5	.2-5	.5-1	1-2	.5-1	1-2	2	5	5-20	.5-1	2	2	5	20
Enterococcus (44062)	1-5	.5	.5-1	1-2	10-20	.5-1	1-2	2	5-10	10-over 50	.5-1	1-2	2-5	10	20-50
<i>Sal. panama</i> (40615)	1-2	1-2	2-5	5-10	10-20	2-5	2-10	10	5-20	10-over 50	5-10	10	10-20	20-50	50-over 50
<i>E. coli</i> (41010)	1-2	2	5	5-20	5-50	2	2-10	10	5-over 50	10-over 50	2-5	5	10	50	Over 50
<i>S. aureus</i> (37215)	.05-2	1-2	1-2	1-2	1-2										
Enterococcus (40056)	1-2	.2-1		.5-1	1-2						.5-1	1-2	2-5	10	20-50

The figures represent the concentrations of aureomycin in  $\mu\text{g}$  per ml, required to inhibit growth.

the magnitude described above as inhibitory were present. No lysis was evident even with concentrations of the agent one hundred times greater than those sufficient for bacteriostasis.

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Four strains of *S. aureus* had been tested against penicillin and streptomycin. Three were penicillin-resistant and one was streptomycin-resistant. All strains were equally sensitive to aureomycin.

**Discussion.** The data presented in this study are in close agreement with those of Lankford and Lacy.<sup>4</sup> Very low concentrations of aureomycin inhibited *S. aureus* if the medium used was plain tryptone broth or tryptone glucose extract agar. The same was true of other organisms tested. Lowest values were obtained with the 4 hour turbidimetric method. Addition of serum or blood to the medium increased the concentration of aureomycin required for inhibition, especially when incubation was prolonged. This effect was quite marked with the plate method. The absence of bactericidal action by aureomycin and the rapid deterioration of this agent during incubation offer an adequate explanation of these observations. A richer medium would be expected to accelerate bacterial growth

TABLE III.  
Aureomycin Sensitivities in  $\mu\text{g}$  per ml. (Tryptone glucose extract agar plate method).

Organism	Hr			
	12	18	24	36
<i>S. aureus</i> (Strains 1, 44488, 44381)	0.2	0.5	0.5	5
<i>Enterococcus</i> (40156)	0.1	—	—	1
<i>Enterococcus</i> (43284)	0.1	0.2	—	—
<i>Enterococcus</i> (45145)	0.2	0.2	0.5	—
<i>E. coli</i> (40382)	1	1-2	—	5
<i>E. coli</i> (44532)	1	5	5	20
<i>E. coli</i> (43768)	1	2	5	20
<i>Ps. aeruginosa</i> (43919)	50	50	50	Over 50

more rapidly as the inhibitory action of the drug declined. Therefore, the higher values reported by other investigators may be ascribed to their use of highly nutritive media, probably containing serum or blood, and to incubation for prolonged periods of time. The supposed direct inactivation of aureomycin by serum or blood reported by Chandler and Bliss<sup>5</sup> may have reflected an interplay of these factors rather than specific action on the drug.

The turbidimetric method with tryptose broth as the medium is probably the most accurate. It has the advantage of permitting rapid determinations, 3 or 4 hours of incubation being sufficient. It is more tedious and exacting than a simple plate method using tryptone agar, which may be preferable for routine laboratory use. Values obtained will be higher than those obtained by the turbidimetric method. Readings should be made within 12 hours of inoculation if possible since the error will be diminished with shorter periods of incubation. A correction factor may be employed, but this will vary widely with the experimental situation even at 12

hours. Eighteen hour determinations may be as much as 25 times greater than the equivalent 4 hour turbidimetric value.

Noting that bacterial sensitivity decreased as the size of the inoculum was increased, Harrell and associates<sup>6</sup> have warned of the potential danger of aureomycin resistance. This possibility has received further support from the detection of viable bacteria in the presence of very high concentrations of the agent. Such organisms were found on subsequent test to be fully sensitive as studied in this laboratory with the turbidimetric method. It is suggested that these bacteria were "persisters."

**Summary.** 1. Gram positive cocci and gram negative bacilli were tested turbidimetrically for aureomycin sensitivity. The inhibitory levels were .02 to .05  $\mu\text{g}$  per ml for *S. aureus*, 0.1  $\mu\text{g}$  per ml for enterococcus, and 0.5  $\mu\text{g}$  per ml for *Sal. panama* and *E. coli*.

2. Aureomycin was not inhibited by the presence of blood or serum in the medium.

3. Increased length of incubation and addition of serum or blood to the medium resulted in higher levels of inhibition, particularly with the plate method. An explanation of this effect is offered.

4. Tryptone glucose extract agar was used for a plate method. Values were higher and less consistent than with the turbidimetric technic

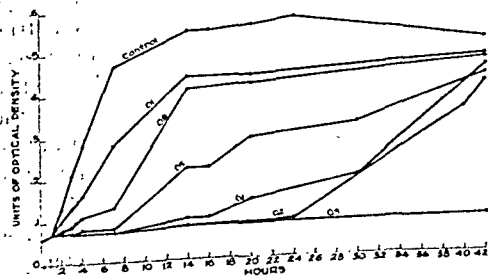


FIG. 1.

*Staph. aureus* growth curves.  
(Aureomycin concentration in micrograms per ml).

<sup>5</sup> Chandler, C. A., and Bliss, E. A., *Ann. New York Acad. Sci.*, 1948, **51**, 221.

<sup>6</sup> Harrell, G. T., Meads, M., and Stevens, K., *South. Med. J.*, 1949, **42**, 4.

## Effect of Androgen on Concentration of Certain Amino Acids in the Rat Prostate.\* (17341)

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*From the University of Texas, M. D. Anderson Hospital for Cancer Research, Houston, Texas.*

Although recent reports<sup>1</sup> have appeared describing the effects of hormones on the biochemical constituents of organs stimulated by those hormones, no information is available relative to the influence of hormones on the free amino acids of such tissues. As part of a large program of growth investigation, it became of interest to make such a study.

Young, adult male rats (140-160 g) of the Sprague-Dawley strain were used. Orchidec-tomy was performed through a mid-ventral incision with the rats under light ether anesthesia. A period of 15 days was allowed for prostatic atrophy. The castrated rats which received androgen were given 0.5 mg of testosterone propionate (Oreton<sup>†</sup>) in 0.25 ml of corn oil subcutaneously each day for periods of 1, 4, 8, and 12 days. All animals were fasted 24 hours, killed by decapitation, and the ventral lobes of the prostates were removed. A sufficient number of glands was pooled to furnish a total of 300-500 mg of tissue. The tissues were prepared and analyzed by paper chromatography according to the method of Awapara.<sup>2</sup> Qualitative separation of the various amino acids was done by the two-dimensional method, and one-dimensional quantitative determinations were carried out for alanine, glycine, glutamic acid, and aspartic acid. The results of the former procedure are shown in Fig. 1, and the values obtained by the latter method are given in Table I.

It can be seen that following castration there are fewer amino acids determinable by

the qualitative technic, and with replacement therapy the variety progressively approaches the normal. This can be interpreted in at least two ways: a) there is an absolute disappearance of certain amino acids, and b) the concentration of these amino acids is reduced below the sensitivity of the method. The latter explanation seems to be the more reasonable. Quantitation of the 4 principal amino acids shows that castration is followed by a marked reduction in their concentrations. It is apparent also that 8-12 days of replacement therapy with 0.5 mg of testosterone propionate each day is necessary before the amino acid concentrations approximate the normal levels. These data cannot be compared with the findings of the authors previously quoted because in those experiments the injections of androgen were begun on the day of castration and, therefore, no time was allowed for atrophy.

In Fig. 1 it can be seen that the normal prostate has at least 4 peptides (14 A, B, C, D) not seen in the glands of castrates or castrates receiving androgen. It is also of interest to note that the prostate of the castrate animal has a substance, probably a peptide, which disappears after 4 days of treatment with androgen. The physiologic significance of these peptides is not at all clear and must await further study. An unidentified substance (Fig. 1, No. 15) was found to be present in every case. Preliminary studies in this laboratory suggest that this substance is cystine.

Androgen replacement stimulates growth and renews the secretory activity of atrophic prostate glands. Since these changes are concomitant with a progressive increase in the concentration of free amino acids, it may be concluded that growth is associated with an increased propensity of tissue for concentrating and retaining these substances. This concept would appear to be important in

\* This work was supported in part by a grant (No. INSTR 23) from the American Cancer Society.

<sup>1</sup> Davis, J. S., Meyer, R. K., and McShan, W. H., *Endocrinology*, 1949, **44**, 1.

<sup>†</sup> The Oreton used was supplied by the Schering Corporation through the courtesy of Dr. Irwin Schwenk.

<sup>2</sup> Awapara, J., *J. Biol. Chem.*, 1949, **178**, 113.

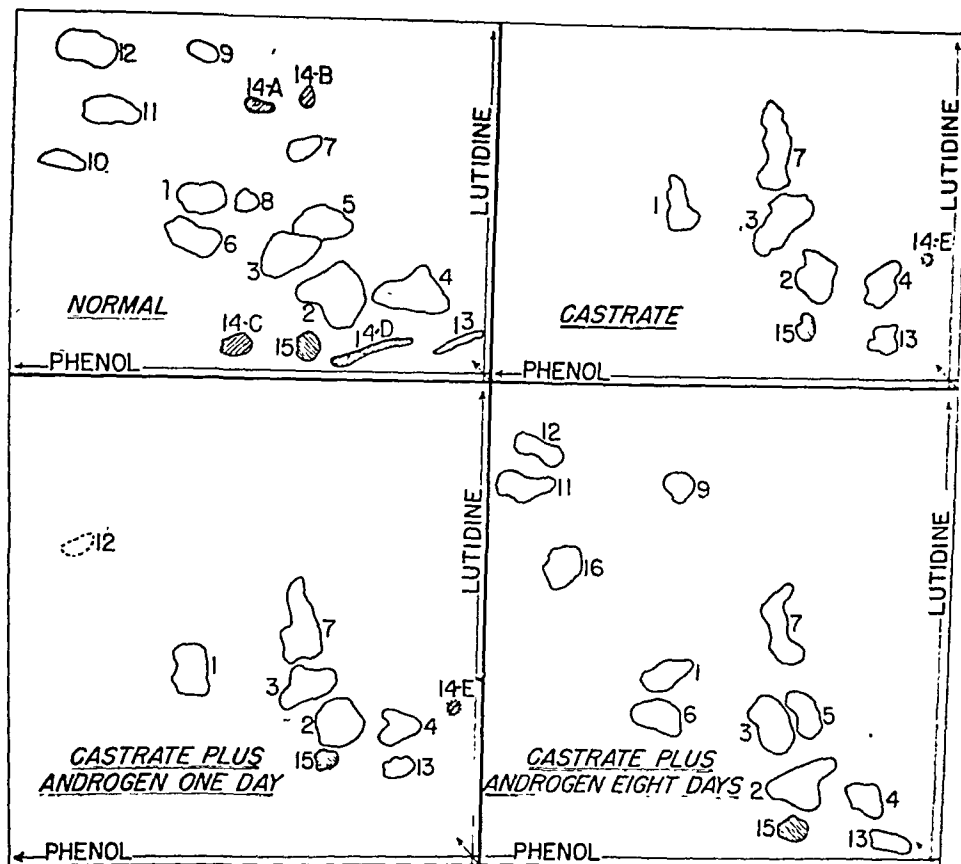


FIG. 1.

Chart showing the distribution of free amino acids as demonstrated by two-dimensional paper chromatography. The numbered areas indicate the positions of the following substances: 1. alanine; 2. glutamic acid; 3. glycine; 4. aspartic acid; 5. serine; 6. glutamine; 7. taurine; 8. threonine; 9. tyrosine; 10. proline; 11. leucine; 12. phenylalanine; 13. glutathione; 14. A, B, C, D, E, unidentified peptides; 15. unidentified (cystine ?); 16. valine.

understanding the stepwise mechanism of protein synthesis in growth. One might question the contribution of prostatic secretion, *per se*, to the increase in amino acid levels. Unpub-

lished data in this laboratory obviate this possibility, in that no free amino acids have been demonstrated in prostatic fluid by the same procedure used above.

TABLE I.  
Effect of Castration and Androgen Replacement on Amino Acids in Rat Prostate.

Treatment	Prostate wt,* mg	Amino acid concentration mg/100 g tissue			
		Aspartic acid	Glutamic acid	Glycine	Alanine
Castrate—15 days	45 (9)	12	16	9	—
Castrate + androgen 24 hr	55 (6)	13	20	12	—
Castrate + androgen 4 days	178 (4)	12	29	16	9
Castrate + androgen 8 days	562 (2)	23	57	25	43
Castrate + androgen 12 days	762 (2)	44	100	44	36
Normal	700 (2)	58	102	45	40

\* The figures in parentheses are the numbers of glands from which the mean weight given was obtained.

**Summary.** A decrease in the amounts and numbers of free amino acids determinable by paper chromatography is associated with prostatic atrophy after castration. Replacement therapy in the castrated animal is followed

by a progressive increase toward normal. The relation of these findings to growth processes is briefly discussed.

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## The Electroencephalogram in Parkinsonism. (17342)

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It is generally recognized that the pathological process in Parkinsonism centers about the basal ganglia. The electrical activity of this region was studied by Meyers and Hayne<sup>1</sup> by direct insertion of insulated electrodes. They found normals to have a striatal rhythm faster than that of the cortex, while patients with Parkinsonism showed sequences of 25 cycle rhythm not recorded from the cortex. Spiegel<sup>2</sup> found the potentials from the cat's thalamus to be much like those from the cortex. Using an electrode driven into the sphenoidal bone Grinker and Serota<sup>3</sup> found 4 to 6 cycle waves as well as alpha rhythm. Using nasopharyngeal electrodes, Barnett<sup>4</sup> failed to find any 4 cycle activity.

Using conventional scalp electrodes, Yeager and Baldes<sup>5</sup> reported 4 cycle waves not synchronous with the tremor in patients with Parkinsonism. Jasper and Andrews<sup>6</sup> found no slow waves in two patients with unilateral Parkinsonism, but did record such activity from 2 patients with advanced bilateral disease. They concluded that the slow activity was present in cortical leads only when tremor was bilateral. Schwab and Cobb<sup>7</sup> recorded

similar potentials from patients with Parkinsonism, but were of the opinion that the slow waves were artifacts from head movement, since they were able to suppress them by mechanically restraining the head. In view of these conflicting opinions, it seemed worth while to investigate the problem further.

The basal leads which we employed consisted of silver cannulae, insulated save at the tip with bakelite varnish, which were inserted, after topical cocaineization, through the nostrils and into the sphenoidal sinuses, where the tips lay against the posterior wall. X-ray examination showed the tips to be in close proximity to the floor of the sella turcica in the majority of cases. In addition to the two sphenoidal leads, scalp electrodes of hypodermic needle type<sup>8</sup> were placed on each side in the frontal, central, and occipital regions, and the two mastoid regions were grounded. The potentials were amplified by a Grass 4 channel electroencephalograph. Recordings were made between the two sphenoidal electrodes, as well as between the sphenoidal electrodes and the pairs of scalp electrodes. Unipolar recordings from the sphenoidal and scalp electrodes were also made.

In general, where both sphenoidal electrodes made good contact, which occurred about half the time, the intersphenoidal potentials resembled most those recorded from the central scalp electrodes, showing mostly 20 cycle ac-

<sup>1</sup> Meyers, Russell, and Hayne, Robert, *Trans. Am. Neurol. Assn.*, 1948, p. 10.

<sup>2</sup> Spiegel, E. A., *Am. J. Physiol.*, 1937, **118**, 569.

<sup>3</sup> Grinker, R. R., and Serota, H., *J. Neurophysiol.*, 1938, **1**, 573.

<sup>4</sup> Barnett, A., *J. Lab. and Clin. Med.*, 1941, **26**, 1659.

<sup>5</sup> Yeager, E. L., and Baldes, E. J., *Proc. Staff Meet. Mayo Cl.*, 1937, **12**, 705.

<sup>6</sup> Jasper, H. H., and Andrew, H. L., *J. Neurophysiol.*, 1938, **1**, 87.

<sup>7</sup> Schwab, R., and Cobb, S., *J. Neurophysiol.*, 1939, **2**, 36.

<sup>8</sup> Newman, H. W., *Stanford Med. Bull.*, 1949, **7**, 61.



tivity of low voltage, with some 10 cycle waves. The potentials recorded between the sphenoidal electrodes and the scalp electrodes corresponded to those secured from the corresponding scalp electrode and ground. Thus there were no waves felt to be characteristic of the sphenoidal leads.

Records were secured from 12 patients with Parkinsonism. Of these, 6 were incidental to cerebral arteriosclerosis and 6 were post-encephalitic. Nine showed gross tremor, bilateral in all cases. Of these patients with tremor, 5 showed no evidence of slow activity on the EEG, either from the sphenoidal or the scalp leads. The remaining 4 showed slow activity, all of them from the sphenoidal leads, and 3 from one or more scalp leads as well. Fig. 1 shows the 4 cycle activity recorded from the sphenoidal and frontal leads in one of these patients, and its disappearance on opening the

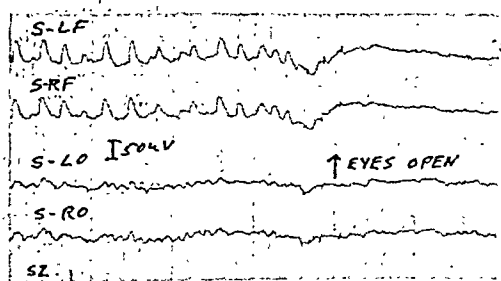


FIG. 1.

Disappearance of 4 cycle activity on opening the eyes in a patient with Parkinsonism. The electrode placement is S-LF, sphenoidal to left frontal; S-RF, sphenoidal to right frontal; S-LO, sphenoidal to left occipital; S-RO, sphenoidal to right occipital.

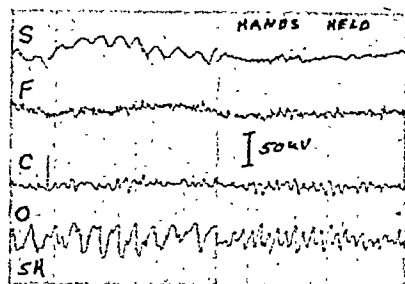


FIG. 2.

Disappearance of 4 cycle rhythm on mechanical restraint of the hands in a patient with Parkinsonism. The electrode placement is S, sphenoidal; F, frontal; C, central, and O, occipital, all bipolar.

eyes, indicating that it was due to eye movement incidental to the tremor. Fig. 2 shows 4 cycle activity in another patient from the sphenoidal and occipital leads, and its disappearance when the patient's hands were restrained.

In the one patient who showed slow activity from the sphenoidal leads but not from the scalp leads it is a little more difficult to dismiss the slow rhythm as artifact, but since it too was suppressed by mechanical restraint it seems reasonable to ascribe it to head movement.

In none of the 3 patients who had no gross tremor was any slow activity demonstrated from either the sphenoidal or scalp leads.

**Summary.** Patients with Parkinsonism do not show slow activity in the EEG, using either sphenoidal or scalp electrodes, except that incidental to movement artifact.

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## Blood Level of Sodium 1-Methyl Butyl Barbituric Acid and Duration of Anesthesia in Rabbits. (17343)

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Since the report of Koppanyi, *et al.*,<sup>1</sup> on the barbiturate-cobalt reaction extensive investi-

<sup>1</sup> Koppanyi, T., Murphy, W. S., and Krop, S., *Proc. Soc. Exp. Biol. and Med.*, 1933, 30, 542.

gations of the metabolic fate of the barbiturates have taken place. The recent development of an ultraviolet spectrophotometric method utilizing small samples of blood has

TABLE I.

Blood Level (mg/%) at Time of Awakening as Influenced by Dose and Route of Administration.

Intravenous, 30 mg/kg	Intramuscular, 35 mg/kg	Intraperitoneal, 35 mg/kg	Subcutaneous, 35 mg/kg	Oral, 50 mg/kg
1.2	1.4	1.2	0.9	1.1
1.1	1.2	1.2	1.6	1.0
1.4	1.1	0.9	1.4	1.1
1.4	1.7	1.2	1.0	1.1
1.1	1.1	1.1	1.6	1.1
1.0	1.0	1.4	1.4	1.7
*1.20	1.25	1.17	1.32	1.12
† .17	.16	.30	.30	.26

\* Mean.

† Standard deviation.

made feasible the reinvestigation of the variations in the blood level of the barbiturate during anesthesia. This report describes the relationship found between the blood level of the barbiturate and the duration of anesthesia.

The sodium salt of 1-methyl butyl barbituric acid was used throughout the study. Female albino rabbits weighing about 2 kg were given a single dose of the drug after having been allowed only water for the 12 to 18 hours preceding injection. Blood samples of 5 cc were obtained by cardiac tap and analyzed by the ultraviolet spectrophotometric method of Goldbaum.<sup>2</sup> Each figure in Table I represents the average of a duplicate analysis. The "awakening time" was taken the instant the animal spontaneously assumes the prone position.

A group of 19 animals was given a dose of 35 mg/kg of sodium pentobarbital intravenously. The injections were made in the marginal vein at the rate of 1 cc (20 mg pentobarbital/cc) per minute. Following the injection the animals were placed in the supine position. Blood samples were withdrawn both at 10 minutes and upon spontaneous righting.

Despite careful attention to the technic of injection there was a wide variation in the level of the drug found in the circulating blood ten minutes after injection. A mean of  $3.21 \pm 0.22$  mg % with a range of from 2.3 to 3.9 mg % was found. In contrast, the level on awakening was constant exhibiting a mean of  $1.16 \pm 0.04$  mg %. The blood level at the time of awakening was found to

be unrelated to the duration of anesthesia. Thus, the shortest time recorded, 45 minutes, was found in an animal with a level on awakening of 1.2 mg %; whereas the animal which slept longest, 225 minutes, had an awakening level of 1.0 mg %. Upon statistical evaluation the correlation coefficient between duration of anesthesia and awakening blood level is found to be  $r = -0.181$ , indicating no dependence. Neither could a relation between the total sleeping time and the concentration of drug in the blood at 10 minutes be established. Although those animals which slept over 200 minutes had 10 minute levels of 3.3 mg % and greater, the correlation coefficient  $r = 0.316$  indicates that no dependent relationship exists.

The effect of route of administration upon the awakening blood level was also investigated. Each of 6 rabbits received 35 mg/kg in the thigh muscle, 6 received 35 mg/kg intraperitoneally, 6 received 35 mg/kg subcutaneously in the flank, and 6 were given 50 mg per kilogram by stomach tube. In addition, 6 rabbits received 30 mg/kg in the marginal ear vein in order to determine the effect of a lower dosage level. The animals were placed on their backs when anesthetized and blood samples were taken as soon as they had righted themselves. The results are shown in Table I.

It will be seen that there was little variation between routes. The mean was found to be about 1.2 mg % for all routes with standard deviations of about 0.2 mg %. Evaluation of the differences between routes by means of the analysis of variance reveals the differences to be of no significance ( $F = 0.38$ ).

<sup>2</sup> Goldbaum, L., *J. Pharm. and Exp. Therap.*, 1948, 94, 68.

*Summary.* 1. The correlation between the blood level of sodium pentobarbital and the duration of anesthesia in rabbits was investigated.

2. The awakening level was found to be quite constant at about 1.2 mg %, and was uninfluenced by route of administration or

dose of the drug in the range tested.

3. No relation could be demonstrated between duration of anesthesia and the barbiturate blood level 10 minutes after injection, or with awakening blood level.

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## A Method of Preparing Collodion Particles for Serologic Agglutination.\* (17344)

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The agglutination of collodion particles as a method of demonstrating union between antigen and antibody has been utilized by several investigators as a highly sensitive serologic technic.<sup>1-3</sup> However, difficulty in producing uniformly active pellets and the frequency of non-specific reactions have limited its general use. Cavelti<sup>4</sup> recently reviewed these points and suggested certain modifications of earlier methods to avoid their inherent difficulties. In the course of attempts made in this laboratory during the past two years to devise a serologic test for viral hepatitis, the use of the collodion particle agglutination technic was explored. A simple method of preparing collodion particles which give consistently reproducible results was devised. It is the object of this paper to describe this method.

*Preparation of collodion particles.* The general method of Loeb<sup>5</sup> modified by Cannon and Marshall<sup>6</sup> was used with certain alterations. All glassware used was sterile.

*Stock solution.* One and one-half pounds

of collodion, U.S.P. Merck (non-flexible) is poured slowly into 2 liters of singly distilled water contained in a 4 liter glass beaker. The water is stirred constantly with a glass rod while the collodion is being added, and a mass of collodion separates. The water is decanted and the mass is then washed three times with distilled water, and is finally pressed by hand between several layers of bibulous paper to remove excess water. The mass of collodion is further dried in an incubator at a temperature of 37°C for 24 hours or until the odor of ether is no longer detectable. During the period of drying, the mass is broken up into smaller pieces to allow exposure of more surface. When dry, the mass of collodion is weighed, and a 5% solution in acetone is prepared in a water bath at a temperature of 37°C. Stirring with a glass rod expedites the solution. This stock solution is stored in the ice-box at 4° to 6°C.

*Suspension of pellets.* 150 ml of stock solution is poured into the glass container of a Waring Blendor. The base of the Blendor is wrapped with a damp towel, and another damp towel is draped over the open top of the glass container to avoid splashing. The apparatus is set before an open window in such a way that escaping fumes of acetone may be readily dispersed by currents of air. A glass funnel with the stem pulled to a fine capillary tip is placed on a ring stand so that the tip is approximately 2 cm below the top

\* These investigations were conducted, in part, with the aid of the Commission on Virus and Rickettsial Diseases, Armed Forces Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D.C.

<sup>1</sup> Goodner, K., *Science*, 1941, 94, 241.

<sup>2</sup> Saslaw, S., and Campbell, C. G., *Proc. Soc. Exp. Biol. and Med.*, 1948, 68, 559.

<sup>3</sup> Lange, K., Gold, M. M. A., Weiner, D., and Simon, V., *J. Clin. Invest.*, 1949, 28, 50.

<sup>4</sup> Cavelti, P. A., *J. Immunol.*, 1947, 57, 141.

<sup>5</sup> Loeb, J., *J. Gen. Physiol.*, 1922, 5, 109.

<sup>6</sup> Cannon, P. R., and Marshall, C. E., *J. Immunol.*, 1940, 38, 365.

of the container and over the vortex of the agitated fluid. The Blender is set in motion, and through the funnel is added 60 ml of a mixture of three parts distilled water and one part acetone. When all the water-acetone mixture has been added, the Blender is turned off, and a heavy gelatinous precipitate separates and settles to the bottom of the glass container. The faintly cloudy supernate is decanted into a filter flask containing 300 cc of cold, doubly distilled water, and the resultant mixture becomes cloudy. The gelatinous precipitate in the glass container is redissolved in 150 ml of stock solution. The Blender is again set in motion, 60 ml of a 3:1 water-acetone mixture is added, and the supernate is subsequently decanted from the gelatinous precipitate into the original filter flask containing the 300 cc of doubly distilled cold water and the first supernate. The gelatinous precipitate remaining in the glass container is redissolved in 100 ml of acetone, agitated with the Blender, and precipitated with proportionate amounts of a 3:1 mixture of water and acetone. This procedure is repeated 2 or 3 times and the resultant supernates are decanted into the original filter flask containing the 300 cc of cold, doubly distilled water and the previous supernates.

The filter flask is then attached to a vacuum pump at a pressure of 25 lb until only a faint odor of acetone remains. This may be expedited by placing the filter flask in a water bath at 56°C. The cloudy suspension in the filter flask is then passed through a thin cotton filter to remove coarse collodion particles. The filtrate is centrifuged in an angle head centrifuge at 4000 r.p.m. for 20 minutes. The faintly cloudy supernate is decanted and discarded. The precipitate is resuspended and washed 3 times in doubly distilled water by centrifuging at a speed of 4000 r.p.m. for 10 minutes. The supernate after the last two washings should be almost clear. The washed particles are resuspended in doubly distilled water and made into a stock suspension of a density standardized so that a 1:10 dilution equals No. 2 of the McFarland Turbidity Scale.<sup>7</sup> Stock suspensions of pellets are kept in the ice-box. They may be stored at this temperature (4° to 6°C.) for

7 to 14 days, after which they tend to lose their capacity to enhance a known serologic response.

The suitability of each lot of pellets for use is determined by testing their capacity to be agglutinated by the interaction of *Pneumococcus* Type I polysaccharide and its homologous hyperimmune horse serum. To chemically clean, sterile, tubes (100 x 10 mm) containing 0.5 ml of polysaccharide in dilutions of  $10^{-4}$  to  $10^{-9}$  are added 0.1 ml of pellets, 0.3 ml of sterile 0.85% saline and 0.1 ml of a 1:5 dilution of hyperimmune horse serum. The mixture is shaken and allowed to stand one hour at room temperature, after which the tubes are spun at 1500 r.p.m. for 5 minutes in a horizontal head centrifuge. The tubes are then flipped, and the amount of agglutination is determined against a bright light with a No. 5 Magni-Focuser glass.<sup>†</sup> A control test is made in the same dilutions with normal horse serum. Characteristically the precipitin test as usually performed with *Pneumococcus* Type I polysaccharide and its homologous hyperimmune horse serum is positive in dilutions of antigen of  $10^{-6}$ . The pellets must be agglutinated (at least 1+) in a dilution of antigen of  $10^{-8}$  for them to be suitable for use as an enhancing mechanism in detecting a serologic response. Many lots of pellets are agglutinated in a dilution of antigen of  $10^{-9}$ . No agglutination occurs in the normal horse serum control tests.

*Summary.* A simple and consistently reproducible method of preparing collodion particles in a Waring Blender is described. Pellets made in this way are uniformly active for 7 to 14 days and give no non-specific reactions in the serologic system tested. In contrast to previously described methods which prohibit the use of metal agitators, the metal blades in the Waring Blender caused no apparent difficulties in the preparation of pellets. Actually, the violence of the agitation produced by the high rotary speed of the blades appeared to assist in the production of more uniformly active and smaller pellets.

<sup>7</sup> McFarland, J., *J.A.M.A.*, 1907, 49, 1176.

<sup>†</sup> Edroy Products Company, New York, N. Y.

## Determination of Serum Antitrypsin in the Study of Malignant Neoplasia. (17345)

MARILYN WALDVOGEL, HORACE N. MARVIN, AND BENJAMIN B. WELLS.\*

*From the University of Texas, M. D. Anderson Hospital for Cancer Research, Houston.*

Before 1920 an antitryptic activity in the blood of various species had been observed, and was the subject of several theories and investigations. Following this there was a period of about 20 years during which the problem was almost entirely neglected. In recent years attention has again been focused on the serum antitrypsin phenomenon, and no less than 10 methods for its study have been reported in the literature. Several of these have been designed for use in the clinical laboratory. Clark, *et al.*,<sup>1</sup> Duthie, *et al.*,<sup>2</sup> Tallan, *et al.*,<sup>3</sup> and others have reported the application of their respective methods to the study of cancer. Although the results obtained by these investigators are in no sense conclusive, there is a prevailing suggestion that serum antitrypsin is a reflection of important physiologic mechanisms and that its alteration in disease should in some manner serve the practical objectives of clinical case study. At least 3 medical problems have been dealt with repeatedly in these reports: (1) endocrine problems, particularly pregnancy and its complications (2) infectious diseases, especially tuberculosis and syphilis, and (3) cancer.

Our interest in serum antitryptic activity persists upon the hope that certain changes may be of value in the diagnosis or study of malignant disease. The method devised in this laboratory<sup>4</sup> has the advantages of speed, simplicity and reproducibility, making it a

suitable tool for extended clinical study. The survey reported herein will summarize our results to date.

**Materials and methods.** Our patients were unselected and included all those admitted to the M.D. Anderson Clinic during the interval of study. Whole blood samples of 3 cc each were drawn and transferred to centrifuge tubes containing 6 mg of crystalline lithium oxalate. The plasma was obtained and serial dilutions with physiological saline were prepared according to the method previously reported.<sup>4</sup> When a large number of plasma samples were to be run simultaneously we found that the procedure could be simplified by using only 4 dilutions (1:40, 1:80, 1:160, and 1:320). The linear segment of the sigmoid curve which expresses the tryptose liberation, and is important in determining the unitage of antitrypsin, almost invariably fell within this range of plasma concentration. Normal, control values were established by determination of the activity in the plasma of individuals who were clinically free of disease.

Of the 174 patients included in this series 81 had some type of malignant neoplasm. In 38 there were known metastases, and in 10 there was concomitant disease. After excluding 15 cases in which the diagnosis was not entirely clear, the remaining 159 were grouped into the several categories shown in Table I. More detailed analysis of the case records failed to reveal other correlations of importance.

**Results.** In the tabulation of our results we have accepted 140 units of antitryptic activity per cubic centimeter of plasma as the upper limit of normal. All our cases of acute infection, pregnancy and fibrocystic disease of the breast showed values in the pathological range. Patients with chronic infections, cardiovascular disease and diabetes showed antitryptic activity within the limits of normal. The malignant diseases were not sharply seg-

\* Present address: Department of Medicine, University of Arkansas School of Medicine, Little Rock, Arkansas.

<sup>1</sup> Clark, D. G. C., Clifton, E. E., and Newton, B. L., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 276.

<sup>2</sup> Duthie, E. S., and Lorenz, L., *Biochem. J.*, 1949, **44**, 167.

<sup>3</sup> Tallan, H. H., Clifton, E. E., and Downie, G. R., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 667.

<sup>4</sup> Wells, B. B., Marvin, H. N., and Waldvogel, M. J., *Am. J. Clin. Path.*, 1949, **10**, 448.

TABLE I.

	Diagnosis	No. cases	Unitage	
			Range	Avg
Antitrypsin <140 units	Non-malignant			
	No disease	52	74-141	112
	Cardiovascular disease	9	69-137	104
	Chronic inflammation	11	76-162	129
	Diabetes	2	127-135	131
	Malignant			
	Terminal malignancy	3	117-123	121
	Chronic myelogenous leukemia	2	132-142	137
	Primary skin cancer	13	98-146	125
	Primary cervical cancer	5	93-142	120
Antitrypsin >140 units	Non-malignant			
	Acute infections	9	158-229	178
	Fibrocystic disease of the breast	2	162-166	164
	Pregnancy	3	166-219	196
	Malignant			
	Primary skin and cervical cancer with acute infection	5	162-193	178
	Primary cancer other than skin and cervix	13	138-204	157
	Acute leukemia	2	155-214	185
	Metastatic cancer of all types	38	151-240	182

regulated on this basis, but there are interesting and well-defined trends. Individuals having extensive malignancies in terminal phase and those having small primary lesions showed no rise in plasma antitrypsin. In the first instance we may suppose that reactivity has been lost and, in the second, that the stimulus was insufficient. Visceral cancer and advanced, but not terminal, malignancy was quite regularly attended by high levels of plasma antitrypsin.

**Discussion.** The antitrypsin content of plasma is not altered in the early and more limited forms of malignant disease. It fails, therefore, to have positive diagnostic value when the condition is most amenable to surgical or radiological intervention. The determination also fails as a means of excluding cancer since terminal, and primary skin and cervical cancer are associated with values within the normal range.

Potentially, the greatest advantage of the procedure may be that the presence of hidden or metastatic cancer is suggested in those patients with more than 140 units of antitrypsin. It is true that in several non-malignant conditions the antitrypsin values are elevated above normal, but these are readily diagnosed by other means. In any event, the underlying

mechanisms of the phenomenon present an interesting field for further investigations.

Both clinical and experimental studies indicate that the plasma antitrypsin concentration is subject to endocrine influence. High levels are regularly found during pregnancy. One is tempted to explain the findings in fibrocystic disease of the breast as a reflection of endocrine imbalance. In this connection we have recently studied a case of seminoma of the testis with pulmonary metastases in which plasma antitrypsin values varied between 219 and 204 units prior to x-ray therapy. During x-ray treatment the antitrypsin decreased rapidly to 147 units while the patient's general condition improved markedly. Values between 147 and 154 were obtained during a period of 6 weeks. At this time stilbestrol therapy was instituted. The plasma antitrypsin increased rapidly to 209 units in less than 3 weeks while the patient showed marked progress of his disease. Since estrogen administration has failed to increase plasma antitrypsin activity in animals we are inclined to attribute these results entirely to fluctuations in growth of the tumor. Plasma antitrypsin in relation to testicular neoplasms deserves considerably more study.

**Conclusions.** 1) The plasma antitrypsin test

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4 Wells, B. B., Marvin, H. N., and Waldvogel, M. J., *Am. J. Clin. Path.*, 1949, **19**, 448.

TABLE I.  
Vitamin B<sub>12</sub> Depletion Diet.

	g/100 g
Purified casein	18
Cystine	0.2
Dextrin	69
Salt mixture No. 1 (U.S.P.)	4
Cellu-flour	2
Agar	1.5
Primex	5
CLO	1
Choline	0.1
Sulfaguanidine	0.5
Protamone (iodinated casein)	0.05
	mg/100 g
Riboflavin	3
Thiamine · HCl	3
Inositol	20
Niacin	3
Pyridoxine · HCl	5
Biotin	.01
Folic acid	.01
Calcium pantothenate	5
p-Amino benzoic acid	5
Menadione	0.5

tended depletion. Groups of about 50 rats are depleted at one time with about 16 rats to a cage. Following depletion, the rats are placed in individual cages with raised floors. Six rats of mixed sex are used for each assay level. As a check on the rat assay, experiments were run, using the dietary regimen and procedure for assay of the animal protein factor (zoopherin), as described by Zucker and Zucker.<sup>5</sup> Following the preferred method described by these authors, we fed diet P60, based on 60% cottonseed meal to the mother rats during gestation and lactation, and to the offspring to 28 days of age when the curative assay was begun. Five male rats were used in each group.

*Oral vs. Injection Administration.* Three vit. B<sub>12</sub> preparations studied were as follows: a concentrate of vit. B<sub>12</sub>, 25 µg per cc, supplied for investigational use by Merck and Company (designated Concentrate No. 1); a concentrate of vit. B<sub>12</sub> prepared in this laboratory (H.H.F.) to contain any desired concentration of vit. B<sub>12</sub> as determined by microbiological assay (designated Concentrate No. 2) and Cobione (Merck), 10 µg crystalline vit. B<sub>12</sub> per cc. Ampoules of Concentrate

No. 1 were used as the standard throughout. A lot of these ampoules was assayed microbiologically by Eleanor Willerton in our laboratories against a lot of material distributed by Merck and Co. as a microbiological assay standard. By repeated comparisons, ampoules of Concentrate No. 1 were found to contain very close to 25 µg of vit. B<sub>12</sub> per cc.

Experiments were devised to compare the activity of vitamin B<sub>12</sub> added directly to the diet and given orally, or by injection, in single or multiple doses. For direct addition to the diet, solutions of vit. B<sub>12</sub> at a level of about 1 µg per cc were mixed with about 20 g of starch. The starch was then dried in air at 50-60°C and added to one kg of the basal diet. Results of assays wherein vit. B<sub>12</sub> was added directly to the diet are shown in Table II.

For comparison of oral versus injection dosage under conditions of multiple dosage, vit. B<sub>12</sub> solutions were made to contain from .33 to 1.0 µg per cc. Intramuscular injections and oral feedings of 0.23 cc per dose were then made 3 times weekly over the 2-week assay period at various assay levels. When comparing the effect of single oral or injection doses, appropriate critical concentrations of B<sub>12</sub> were administered the first day of assay corresponding to a range of .025 to .075 µg per rat day for the 14-day period. The results of the different modes of administration are shown in Table III.

Experiments were designed also to relate the activity of vitamin B<sub>12</sub> in a very preliminary way with the labeled A.P.A. potency of liver extract preparations of known clinical activity. The microbiological assay method of Skeggs, Huff, Wright and Bosshardt<sup>6</sup> provided a further basis for correlation. Aseptic addition of the liver extract supplements to the microbiological medium was used to avoid destruction of the vit. B<sub>12</sub> which, according to Stokstad *et al.*,<sup>7</sup> occurs on autoclaving.

The results of the comparison assays be-

<sup>6</sup> Skeggs, H. R., Huff, J. W., Wright, L. D., and Bosshardt, D. K., *J. Biol. Chem.*, 1948, **170**, 1459.

<sup>7</sup> Stokstad, E. L. R., Dornbush, A. C., Franklin, A. L., Hoffman, C. E., Hutehings, B. L., and Jukes, T. H., *Fed. Proc.*, 1949, **8**, 257.

<sup>5</sup> Zucker, L. M., and Zucker, T. F., *Arch. Biochem.*, 1948, **16**, 115.



does not show changes in association with early or minimal lesions of cancer.

2) The test may aid in excluding the diagnosis of advanced but non-terminal malignancy, or in the discovery of these lesions when they present themselves in obscure form.

3) Since antitrypsin values appear to be closely related to the growth of seminoma of the testis, it suggested that the test may have unique prognostic value in the study of this condition.

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### Rat Growth Assay for Vitamin B<sub>12</sub>. (17346)

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The method of assay for vitamin B<sub>12</sub> herein described was developed using a diet based on purified casein. The diet was made to contain 0.05% iodinated casein (Protamone) as a metabolic stimulant and 0.5% sulfaguanidine to inhibit bacterial synthesis of vitamins. Weanling rats, depleted on this diet for about 7 days, show a depression of growth rate which is counteracted by administration of vitamin B<sub>12</sub>. The rate of response within a critical range is proportional to the amount of vitamin B<sub>12</sub> administered, either as the purified vitamin or as concentrates of the vitamin, such as liver extract.

It is well known that liver extract and vit. B<sub>12</sub> show much higher activity by injection than by oral administration in the pernicious anemia patient. We were interested in the present study to determine whether a difference could be shown between the effects of oral and intramuscular administration of the vitamin. We were interested also to determine the comparative effects of single doses at the beginning of the assay period versus divided doses throughout the assay period. Following completion of these studies, Emerson<sup>1</sup> reported that vitamin B<sub>12</sub> is equally active by either the oral or subcutaneous route in daily doses ranging from 0.0625 to 0.5 µg per rat day, somewhat higher than the range we have studied.

Register, Ruegamer and Elvehjem<sup>2</sup> reported

a quantitative response in rat growth in their assay in the range of 0.025 to 0.1 U.S.P. units per rat day in the form of commercial liver extract. Direct comparison with vit. B<sub>12</sub> activity was not made in the Wisconsin report. We were interested to determine with a sample liver extract whether 1 µg of vit. B<sub>12</sub> would correspond fairly closely by rat assay to 1 U.S.P. unit, a figure previously suggested by Rickes *et al.*,<sup>3</sup> to be in approximately the correct range.

*Experimental.* Weanling rats, 35-45 g, of mixed sex were placed on a vitamin B<sub>12</sub> low diet for depletion. The B<sub>12</sub> depletion diet has the composition shown in Table I. In preliminary assays S.M.A vitamin test casein was further purified by the method of solution and reprecipitation of Novak and Hauge.<sup>4</sup> Later we found that vitamin test casein gave satisfactory results without further purification.

The rate of weight gain is about 12-15 g during the second week on the depletion diet and diminishes to about one-half this rate during the third week of depletion. The depletion period used ranged from 7 to 14 days. The 7-day period is preferred because of the increased mortality which occurs on more ex-

<sup>2</sup> Register, U. D., Ruegamer, W. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, **177**, 129.

<sup>3</sup> Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K., *Science*, 1948, **107**, 396.

<sup>4</sup> Novak, A. F., and Hauge, S. M., *J. Biol. Chem.*, 1947, **174**, 647.

<sup>1</sup> Emerson, G. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 392.

TABLE IV.  
Vitamin B<sub>12</sub> Rat Assay—Liver Extracts.

Exp.	Supplement	Avg wt gain and range		
		2 wk, g	3 wk, g	4 wk, g
Assay method of Zucker and Zucker	None	44	54	74 (59-84)
	Vit. B <sub>12</sub> conc. No. 1, 5 µg/kg diet	63	80	100 (85-128)
	Liver extr. No. 1, 5 U.S.P. units/kg diet	65	80	98 (78-116)
		Avg 2 wk wt gain and stand. error, g		
Method in study	None	17.7 ± 2.7		
	Vit. B <sub>12</sub> conc. No. 1, = .05 µg daily (i.m.)*	36.8 ± 4.4		
	Liver extr. No. 1, + .05 U.S.P. unit daily (i.m.)*	34.6 ± 3.6		
Method in study	None	17.7 ± 3.7		
	Vit. B <sub>12</sub> conc. No. 1, = .1 µg/day	41.1 ± 3.3		
	Liver extr. No. 1 = 0.1 U.S.P. unit/day (i.m.)*	44.8 ± 5		
	(Equal to .1 µg B <sub>12</sub> by <i>L. leichmannii</i> assay)			
	Liver extr. No. 2 = 0.2 U.S.P. unit/day (i.m.)*	34.5 ± 4.2		
		(Equal to .044 µg B <sub>12</sub> by <i>L. leichmannii</i> assay)		

\* Injections made intramuscularly 3 times per week in doses to supply the daily equivalent shown.

from outside sources have not been tried.

The depletion period following weaning must remain, to some extent, a matter of judgment, depending on the condition of various lots of animals. It appears necessary to deplete the animals until the controls gain an average of only about 20 g during the 2-week period of assay. This has held true with a 7-14 day depletion in all but one of 12 assays in this laboratory involving about 600 rats.

No significant difference has been noted between the response of male and female rats in these studies, and precautions other than equal pairing as to sex do not appear necessary or desirable on the basis of convenience. Although there is considerable variation in response among individual animals, as shown by the fairly high standard errors (Tables II-IV), the significance of the results also appears high because of the distinct spread in increments between the control and test groups. In a few instances assays have been extended for 3 weeks. In these cases the animals supplemented with vit. B<sub>12</sub> continued to gain at a steady rate, whereas the control animals showed a small and constantly declining rate of weight gain, plus an increasing mortality. Occasional control rats gain weight rapidly without any supplement. We surmise that these unusual animals have

a high rate of intestinal synthesis of vit. B<sub>12</sub>, similar to the fast growing rats described by Hartman, Dryden and Cary,<sup>8</sup> and that neither the sulfaguanidine nor iodinated casein affect these particular rats nearly as much as they do the general run of test animals. In the above-mentioned single assay run in which the animals were not sufficiently depleted before assay, the control group gained weight nearly as rapidly the first 2 weeks as the supplemented groups. The groups were continued another week during which the supplemented groups gained considerably more weight than the controls, indicating that depletion of the control group had finally taken effect.

Our findings are in accord with those of Lillie, Denton and Bird<sup>9</sup> with chicks indicating that a single administration of vitamin B<sub>12</sub> at the beginning of assay meets the needs of deficient animals for growth for at least an ensuing 2-week period. Thus the assay may be simplified to involve only one administration of concentrated materials, such as liver extract, which lend themselves to this technic. An advantage of the single dosage technic is found in supplying the deficient

<sup>8</sup> Hartman, A. M., Dryden, L. P., and Cary, C. A., *Fed. Proc.*, 1949, **8**, 205.

<sup>9</sup> Lillie, R. J., Denton, C. A., and Bird, H. R., *J. Biol. Chem.*, 1948, **176**, 1477.

RAT GROWTH ASSAY FOR VITAMIN B<sub>12</sub>
 TABLE II.  
 Direct Addition of Vitamin B<sub>12</sub> to the Diet.

Exp.	Supplement	Vit. B <sub>12</sub> * per kg diet, μg	Avg 2 wk wt gain and std. error,† g
1	None	0	21†
	Cobione	5	39 ± 3.5
	"	10	53 ± 3.7
	"	30	49.4 ± 4.2
2	None	0	17.6 ± 6.3
	Vit. B <sub>12</sub> concentrate No. 1	5	25.3 ± 0.2
	Vit. B <sub>12</sub> concentrate No. 1	15	39.3 ± 5.5
3	None	0	18.1 ± 3.1
	Vit. B <sub>12</sub> concentrate No. 1	10	38.5 ± 3.9
	Vit. B <sub>12</sub> concentrate No. 2	10	37.2 ± 4.3
	Vit. B <sub>12</sub> concentrate No. 2a	10	40.6 ± 2.2

\* The preparations used were checked for vitamin B<sub>12</sub> content by microbiological assay, one against the other, and against a Merek microbiological assay standard. See text.

$$\dagger \text{Standard error} = \frac{\sqrt{\sum d^2}}{\sqrt{n(n-1)}}$$

‡ Only 3 of 6 rats survived.

 TABLE III.  
 Vitamin B<sub>12</sub> Assays—Injection and Oral Administration.

Exp.	Supplement	Avg 4 wk wt gain and range, g
Assay method of Zucker and Zucker	None	35.4 (31-41)
	Vit. B <sub>12</sub> conc. No. 1, 10 μg per kg diet	123.2 (113-140)
	Vit. B <sub>12</sub> conc. No. 1, = .05 μg daily by mouth*	110.1 (90-138)
	Vit. B <sub>12</sub> conc. No. 1, = .05 μg daily by inj.*	123.6 (108-140)
	Vit. B <sub>12</sub> conc. No. 2, = .05 μg daily by inj.*	115.0 (110-120)
Method in study	None	Avg 2 wk wt gain and stand. error 18.2 ± 4.5
	Vit. B <sub>12</sub> conc. No. 1, 0.35 μg inj. (single dose)	26.0 ± 2.9
	Vit. B <sub>12</sub> conc. No. 1, 0.35 μg oral (single dose)	28.5 ± 5.5
	None	17.7 ± 3.7
Method in study	Vit. B <sub>12</sub> conc. No. 1, = .033 μg daily by inj.*	27.3 ± 9
	Vit. B <sub>12</sub> conc. No. 1, = .067 μg daily by inj.*	35 ± 3.7
	Vit. B <sub>12</sub> conc. No. 2, = .1 μg daily by inj.*	41.1 ± 3.3
	Vit. B <sub>12</sub> conc. No. 2, = .1 μg daily by inj.*	41.1 ± 3.3

\* Injections and oral dosings—3 times weekly to supply daily equivalent shown.

tween vit. B<sub>12</sub> and 2 injectable liver extract preparations are shown in Table IV. The microbiological assay of Liver Extract No. 1 indicated a vit. B<sub>12</sub> content of one μg per U.S.P. unit. This value appears to be confirmed by the rat assay. Liver Extract No. 2, which showed a minimal clinical response, was found to contain only .22 μg of vit. B<sub>12</sub> per estimated U.S.P. unit by microbiological assay. Good agreement with this low value was indicated by the rat assay response. A resumé

of experience in this laboratory in the preparation of experimental liver extracts for clinical trial has indicated in general that preparations low in vit. B<sub>12</sub> are correspondingly low in clinical activity.

*Discussion.* An advantage of the method described herein is the ability to use animals weaned directly from mothers on stock diet, without special dietary control of the parent animals. This is true at least for animals reared in our own stock colony. Weanling rats

ior of this material.

**Methods. Preparation of materials.** The mucoprotein fractions were isolated in the manner already described from perchloric acid filtrates of pooled, normal, human plasma, and in one case (Preparation No. 44) from the plasma of a patient with gastric carcinoma whose plasma mucoprotein-tyrosine level was 4.9 mg % in comparison to normal levels of 2-4 mg %.<sup>2</sup> We are indebted to Dr. M. P. Petermann of the Memorial Hospital of New York City for the lyophilized sample of this plasma. Preparations were thoroughly dialyzed after isolation, and were lyophilized.

**Chemical characterization.** The lyophilized samples were analyzed for carbohydrate, glucosamine, nitrogen, and tyrosine<sup>†</sup> by the methods previously described.<sup>1</sup>

**Electrophoresis.** Samples were prepared for electrophoresis by dissolving them in the desired buffer, with subsequent dialysis against two portions of buffer for a period of at least 24 hours in each portion of buffer. The temperature was 5°C during the dialysis.

In most cases a small amount of insoluble material was present, and solutions were centrifuged immediately before electrophoresis. In order to cover a wide range of pH values with each preparation, it was necessary to recover material from the electrophoresis cell and use it in subsequent measurements. Recovered solutions were concentrated by placing them in a dialysis bag and blowing air over the bag at room temperature. This procedure, and the limited amounts of material available, made it impractical to control the concentrations, so that there was considerable variation in protein concentration in different experiments. It also seems probable that some change in composition took place during repeated recovery and concentration. This has contributed to the difficulty of identifying components at different pH values in some instances.

The buffer systems were prepared with diethyl barbiturate, acetate, citrate or phos-

phate. All buffers were made to give a final concentration of 0.08 M NaCl and 0.02 M with respect to the monosodium salt of the buffer system or the disodium salt in the case of phosphate. HCl was added to obtain the desired pH, so that the ionic strength was 0.1 in each case except at pH 6, where disodium phosphate was employed. In this case the ionic strength was correspondingly greater.

Electrophoresis was carried out in an 11 ml Tiselius cell at 2.0°C, with a potential gradient of between 5 and 6 volts per cm. The conductivities used to calculate the potential gradient were also determined at 2°C on the buffer used to fill the electrophoresis cell. The pH of the buffer, also determined after dialysis, was obtained with a Beckman pH meter at room temperature.

The boundaries were photographed, generally after 3 hours, using the Philpot optical system,<sup>3</sup> and at a magnification of 1.05 to 1. The photographs were enlarged somewhat over 2 times for making the required measurements.

**Experimental results.** The preparations used in this study had the chemical characteristics shown in Table I. These preparations show the characteristically high carbohydrate and glucosamine content previously reported.

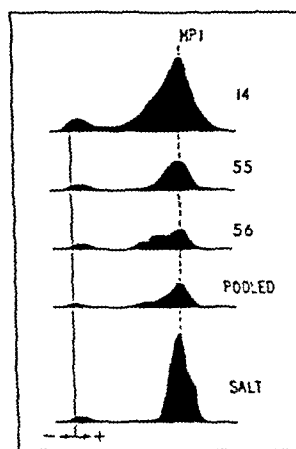


FIG. 1.

Electrophoresis patterns of mucoprotein preparations at pH 8.4. Descending boundaries. Preparations No. 14, No. 55, No. 56, and "pooled," are from perchloric acid filtrates of pooled, normal plasma. The "Salt" preparation was prepared entirely by ammonium sulfate fractionation, and contains 31% albumin.

<sup>2</sup> Winzler, R. J., and Smyth, I. M., *J. Clin. Invest.*, 1948, 27, 617.

<sup>†</sup> Although reported as tyrosine, it is recognized that the Folin's phenol reagent used is not specific for this amino acid.

<sup>3</sup> Philpot, J. S. L., *Nature*, 1938, 141, 283.

animals the full supplement at a time most conducive to overcoming the acute phase of the deficiency.

The present data indicate that vit. B<sub>12</sub> is about equally effective by oral or injection administration in promoting growth of young rats on highly specialized depletion diets. The deficiency of vit. B<sub>12</sub> induced in rats by these rather extreme dietary means does not, therefore, appear to evoke a failure in utilization of the vitamin, but rather a true deficiency and enhanced need for the vitamin itself.

The following additions to the diet failed to yield a comparable growth response, and even appeared to have an inhibitory effect: 5-10% purified casein, 5-10% of various primary-grown and brewer's yeasts, and additional folic acid. The addition of thymidine, 25-50 mg per kg diet, betaine hydrochloride 0.5%, or additional choline 0.1% likewise did not stimulate growth in absence of vit. B<sub>12</sub>.

The growth promoting action of vit. B<sub>12</sub> in rations containing iodinated protein has recently been reviewed.<sup>10</sup> Bethel and Lardy<sup>11</sup> have further compared the effectiveness of vit. B<sub>12</sub>, whole liver substance and extracts high in APA activity, as growth promoting materials for hyperthyroid rats. The ration used by these authors was also based on puri-

fied casein, but did not contain a sulfa drug. It is worthy of note that mortality is high among our control animals and that death is generally preceded by appearance of hemorrhage about the nose and paws. Vit. B<sub>12</sub> clearly prevents this syndrome and has a marked effect toward longevity under the dietary conditions imposed.

The question of the equivalence of vit. B<sub>12</sub> with U.S.P. anti-pernicious anemia units can only be answered by assays in human pernicious anemia patients. Microbiological and animal assays may serve as a useful guide, however, toward this goal. The data presented provide some evidence that concentrated liver extracts which show a correlation by microbiological assay of 1 U.S.P. unit approximately equal to 1  $\mu$ g of vit. B<sub>12</sub>, show a similar approximate equivalence by rat assay.

**Summary.** The growth response of vit. B<sub>12</sub> depleted rats under the conditions studied is proportional to the vit. B<sub>12</sub> administered in the critical range of .025-.1  $\mu$ g per rat day. Oral and injection administration of the vitamin at critical levels yield approximately equal growth responses. Addition of the vitamin to the diet, multiple dosing or single dosing the first day of assay also appear roughly equivalent. A preliminary correlation between the results of microbiological and rat assays as applied to injectable liver extracts was obtained.

Received August 4, 1949. P.S.E.B.M., 1949, 72.

<sup>10</sup> *Nutrition Reviews*, 1949, 7, 183.

<sup>11</sup> Bethel, J. J., and Lardy, H. A., *J. Nutrition*, 1949, 37, 495.

### Mucoproteins of Human Plasma. III. Electrophoretic Studies of Mucoproteins from Perchloric Acid Filtrates of Plasma.\* (17347)

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It has been shown<sup>1</sup> that the "protease" of

human plasma is a mixture of mucoproteins with isoelectric points which are low in comparison with those of the principal plasma proteins. The present communication deals in more detail with the electrophoretic behavior

\* The electrophoresis studies were supported by a grant from the United States Public Health Service and the isolation studies were supported by the American Cancer Society. Contribution No. 224 from the Department of Biochemistry and Nutrition, University of Southern California, School of Medicine.

<sup>1</sup> Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M., *J. Clin. Invest.*, 1948, 27, 609.

TABLE I.

Preparation	Source	Yield mg/100 ml plasma	Nitrogen, %	Glucosamine, %	Carbohydrate, %	Tyrosine, %
No. 14	Human, normal	38	8.1	10.1	15.6	3.9
No. 44	" cancer	100	7.5	8.9	16.3	3.9
No. 55	Human, normal	28	9.0	9.1	15.7	4.3
No. 56	" "	32	7.1	8.1	13.6	3.4
Pooled*	" "	—	8.3	9.8	14.3	3.6
Salt fract.†	" "	72	7.25	7.6	13.2	3.1

\* Five separately isolated lots were pooled for this sample.

† Contained 31% albumin by electrophoresis, assuming equal refractive increments per g of albumin and MP-1.

about 2.3 in citrate buffer.

At pH 8.4, some material slower than MP-1 is always seen, and at pH 4.5 there is generally a considerable amount of material with a mobility of about  $-2.3 \times 10^{-5}$ . This material which we have designated MP-2, has an isoelectric point at about pH 3.4. From the analytical data, it must also be a mucoprotein.

The material designated as MP-3 might conceivably be contaminated with albumin. The mobility is not very different from that of albumin in this buffer, and the broad peaks for MP-3 would generally overlap the mobility for albumin at pH 4.5. Small amounts of albumin might also contribute to the broadening of the electrophoretic peaks at pH 8.4. However, preparation No. 56 could not contain enough albumin to account for the peak observed at pH 4.5. Pending the isolation of more homogeneous preparations, the status of this component must remain in considerable doubt. The isoelectric point, however, is in the region of pH 4.3.

It is clear from the study of these preparations that mucoprotein components MP-1 and MP-2 should be demonstrable by electrophoresis of plasma at a suitable, more acid pH than is generally employed. Such a study has been made by Petermann *et al.*,<sup>4</sup> and further studies in this direction are being presented in paper IV of this series.<sup>5</sup>

The sample isolated from a patient with

cancer (No. 44) presented essentially the same characteristics on electrophoresis as did the pooled sample. The similarity with respect to proportion of components was greater than the analytical data would have led one to suspect. This case, at least, would indicate that the increased amount of mucoprotein in cancer represents an increase in normal components rather than the appearance of an abnormal component.

Although the groups responsible for the low isoelectric points of these mucoproteins have not been identified, it seems likely that they are at least in part sulfuric acid ester groups. Sulfur in excess of that accounted for by cystine and methionine has been observed in preparations of mucoprotein from rat blood,<sup>6</sup> horse serum,<sup>7</sup> and human plasma.<sup>1</sup>

**Summary.** The electrophoretic behavior of mucoproteins isolated from perchloric acid filtrates of human plasma has been studied over the pH range of 2 to 8.4. There are at least 3 electrophoretic components in such preparations, the isoelectric points being approximately 2.3, 3.4 and 4.3. The major component has the lowest isoelectric point and travels with a mobility characteristic of  $\alpha_1$ -globulin at pH 8.4. The increase in the mucoprotein level isolated from the plasma of a patient with gastric cancer was largely in this acid mucoprotein fraction.

<sup>6</sup> Winzler, R. J., and Burk, D., *J. Nat. Cancer Inst.*, 1944, **4**, 417.

<sup>7</sup> Mayer, K., *Z. f. Physiol. Chem.*, 1942, **275**, 16.

Received August 4, 1949. P.S.E.B.M., 1949, **72**.

<sup>4</sup> Petermann, M. P., Karnovsky, D. A., and Hogness, K. R., *Cancer*, 1948, **1**, 104.

<sup>5</sup> Mehl, J. W., Golden, F., and Winzler, R. J., *Proc. Soc. Exp. Biol. and Med.*, 1949, **72**, 110.

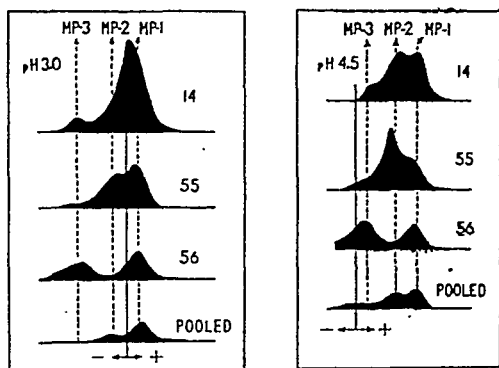


FIG. 2.

Electrophoresis patterns of mucoprotein preparations at pH 3 and 4.5. Boundaries descending toward the anode.

The electrophoresis patterns of the descending boundaries obtained on some of the preparations from human plasma are reproduced in Fig. 1 and 2. In the patterns of Fig. 1, obtained at pH 8.4, is included the pattern of a preparation made entirely by ammonium sulfate fractionation without preliminary deproteinization with perchloric acid. Although this preparation contains 31% albumin, it does demonstrate the greater degree of homogeneity which can be obtained with respect to the major mucoprotein component (MP-1) by such a fractionation procedure.<sup>‡</sup> In Fig. 2, obtained at pH 3.0 and 4.5, are indicated components which we have designated as MP-1, MP-2, and MP-3. At pH 6 and higher, the resolution into well-defined components is less satisfactory and we have only indicated the position of MP-1 in Fig. 1.

In Fig. 3 we have attempted to present the pH-mobility curves for the descending boundaries of these components. Although we feel reasonably well satisfied with the general characteristics in the region below a pH of about 5.5, the uncertainty regarding MP-2 and MP-3 at higher pH's has been indicated in the figure.

**Discussion.** Human plasma mucoprotein prepared by the preliminary removal of other plasma proteins with perchloric acid may contain at least 3 electrophoretically different

components, and the spreading of the boundaries suggests that the degree of heterogeneity is even greater than this. The proportions of the components vary considerably from preparation to preparation, and despite the rather drastic initial treatment with perchloric acid the preparations may be somewhat modified when solutions are repeatedly recovered by evaporation at room temperature. Because of these limitations, conclusions must be drawn with some caution. The preparations labeled No. 14, No. 55, and "pooled" are the most typical of preparations which we have presented for discussion. The analytical values which are given in Table I are in reasonably good agreement for these three. Preparation No. 56 had the least typical electrophoretic behavior and had the lowest values for glucosamine, carbohydrate, and tyrosine, except for the material prepared exclusively by salt fractionation, which contained 31 per cent albumin.

The one component which is seen in all preparations, including the "salt" preparation is MP-1. This material has a mobility of  $-6.4 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1} \text{ volt}^{-1}$  in the descending boundary at pH 8.4, and would appear in the  $\alpha_1$ -globulin fraction of serum at this pH. This component is still strongly negatively charged at pH 4.5, and is isoelectric at a pH of

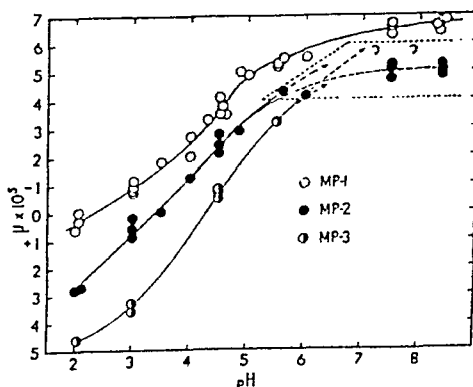


FIG. 3.

Mobility of mucoprotein components as a function of pH. Identification of components other than MP-1 is uncertain above pH 6, and this area of uncertainty is indicated by enclosing it in the dotted lines. Principally because of the spread of peaks, mobilities at all pH values are subject to considerable error.

<sup>‡</sup> This preparation was made by Mr. Henry Weimer, and results of further refinements of this method will be reported in the near future.

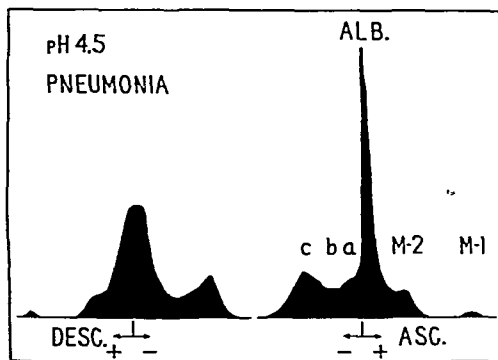


FIG. 2.

Electrophoresis patterns, ascending and descending, at pH 4.5, of serum from a patient with pneumonia. The designation of the components is shown.

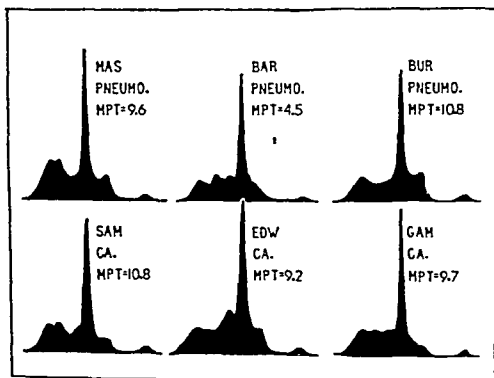


FIG. 3.

Electrophoresis patterns, descending to the cathode, at pH 4.5. The 3 upper patterns are from patients with pneumonia, the 3 lower patterns from patients with cancer. The value for MPT is that for the mucoprotein-tyrosine in mg%.

areas of boundaries in the electrophoresis pattern.

**Experimental results. Electrophoresis at pH 8.4.** In order to show that the mucoprotein does contribute to the  $\alpha_1$ -globulin fraction, a sample of normal plasma was subjected to electrophoresis with and without added mucoprotein. The mucoprotein fraction used for this study was one isolated by ammonium sulfate fractionation and consisted only of the fraction designated as MP-1,<sup>3</sup> contaminated with some albumin. The electrophoresis patterns obtained in this experiment are shown in Fig. 1-A and 1-B, and it is apparent that the mucoprotein MP-1 does indeed increase the  $\alpha_1$ -globulin fraction at pH 8.4.

**Electrophoresis at pH 4.5.** On the basis of the results obtained with the isolated mucoprotein, a pH of 4.5 was selected as being suitable for the demonstration of the mucoprotein in serum. In Fig. 2 are reproduced the ascending and descending boundaries obtained in the electrophoresis of serum from a patient (HUM) with pneumonia, indicating the division into components which we have employed, and their designations. We have designated the 2 acid components as M-1 and M-2 and the globulins as a, b, and c. The separation of the peaks is generally better in the boundary in which the globulins are descending and mucoprotein is ascending and only this boundary is given in the electrophoresis patterns of Fig. 1 and 3. Although the example in Fig. 2 is not the most satisfactory for illustrating the presence of components a, b, and c, an inspection of the patterns in Fig. 3 will indicate that such a division is justified. The acid components M-1 and M-2 are clearly evident in Fig. 2. The amounts of M-1 and M-2 are considerably elevated over the normal levels (compare Fig. 1-C). This has been true of all cases studied in which the chemical determination showed an elevation of the mucoprotein.

The electrophoresis pattern of the same normal serum which was used at pH 8.4 is shown at pH 4.5 in Fig. 1-C. As with the pathological serum of Fig. 2, there are two components corresponding to M-1 and M-2 but in considerably smaller quantities. In Fig. 1-D is shown the electrophoresis pattern at pH 4.5 of this same normal serum with the addition of the same mucoprotein preparation employed at pH 8.4. M-1 is increased, and M-2 is unchanged, suggesting that the MP-1 fraction of isolated mucoprotein may be identified with the M-1 fraction in serum. The mobility of  $-4.2 \times 10^{-5}$  (in the ascending mucoprotein boundary) is in good agreement with that observed in the isolated mucoprotein.

In Fig. 1-E the electrophoresis pattern of serum from a patient with cancer is presented, and in Fig. 1-F the same serum with mucoprotein added. In this case, the mucoprotein was isolated from a perchloric acid filtrate of plasma by the method previously de-



# Mucoproteins of Human Plasma. IV. Electrophoretic Demonstration of Mucoproteins in Serum at pH 4.5.\* (17348)

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Mucoproteins in human plasma have been isolated and characterized chemically,<sup>1</sup> their increase in cancer has been discussed,<sup>2</sup> and the material isolated from plasma has been studied electrophoretically.<sup>3</sup> It was shown that in the usual electrophoresis pattern at pH 8.4 much of the mucoprotein should migrate with the  $\alpha_1$ -globulin, and would thus be included with any other material which contributes to the  $\alpha_1$ -globulin fraction. It was found, however, that the isoelectric points of the fractions contributing to the isolated mucoprotein were quite low, and that they would all remain negatively charged at pH 4.5. Such a pH would, then, appear more suitable for the electrophoretic demonstration of these mucoproteins in serum. It has been shown by Petermann *et al.*<sup>4</sup> that there is an acid component in human serum which may be demonstrated by electrophoresis at pH 4.0, and which increases in amount in those conditions in which the mucoprotein has been shown to be increased. They have suggested that this electrophoretic component may be the same mucoprotein as that with which we have been concerned, and the present studies were intended primarily to determine the extent to which such an identification can be made.

**Methods.** The methods employed in the electrophoresis studies were the same as those

previously described.<sup>3</sup> All electrophoresis measurements were made at 2°C at an ionic strength of 0.1. At pH 8.4, the buffer was diethyl barbiturate, and at pH 4.5 the buffer was acetate. In both cases, the ion and undissociated acid of the buffer had a total concentration of 0.02 M. The pH is that determined at room temperature. Serum samples were generally diluted to 6 times their original volume, and thus had a total protein concentration in the neighborhood of one per cent. The patterns were photographed after 3 hours.

At pH 4.5, some precipitate forms during dialysis, and is removed before electrophoresis. The nature of this precipitate and its effect upon the electrophoresis pattern has not been thoroughly investigated, but the amounts of protein involved are small. Where protein concentrations are reported subsequently, they have been calculated from the total protein concentration of the serum, and the relative

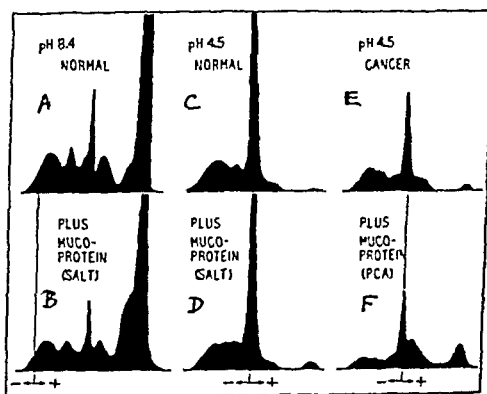


FIG. 1.

Electrophoresis patterns at pH 8.4 and pH 4.5. Boundaries descending to anode at pH 8.4, descending to cathode at pH 4.5.

A. Normal plasma at pH 8.4. B. Same normal plasma at pH 8.4 with mucoprotein (MP-1) added. C. Same normal plasma at pH 4.5. D. Same normal plasma at pH 4.5 with mucoprotein (MP-1) added. E. Serum from cancer patient at pH 4.5. F. Same cancer serum at pH 4.5, with mucoprotein added (MP-1, MP-2, and MP-3).

\* This study was supported by a grant from the U. S. Public Health Service. Contribution No. 225 from the Department of Biochemistry and Nutrition, University of Southern California School of Medicine.

1 Winzler, R. J., Devor, A. W., Mehl, J. W., and Smyth, I. M., *J. Clin. Invest.*, 1948, **27**, 609.

2 Winzler, R. J., and Smyth, I. M., *J. Clin. Invest.*, 1948, **27**, 617.

3 Mehl, J. W., Humphrey, J., and Winzler, R. J., *Proc. Soc. Exp. Biol. and Med.*, 1949, **72**, 106.

4 Petermann, M. P., Karnovsky, D. A., and Hogness, K. R., *Cancer*, 1948, **1**, 104.

TABLE II.

Electrophoretic Analysis of the Sera of 3 Patients, GAM, BUR, and HUM. The concentrations are calculated from the total serum protein, assuming that the refractive index increment is the same for all components. The mucoprotein nitrogen represents the difference between that in perchloric acid and tungstic acid filtrates.\*

pH	Component	Concentration g/100 cc		
		GAM	HUM	BUR
8.4	Albumin	3.3	2.9	2.2
	$\alpha_1$ -globulin	0.3	0.6	0.4
	$\alpha_2$ - " *	—	—	0.8
	$\beta$ - " "	1.0	1.0	1.1
	$\gamma$ - " "	0.6	0.6	0.7
	" "	0.9	1.2	1.3
4.5	M-1	0.3	0.1	0.2
	M-2	0.4	0.5	0.8
	Albumin	2.9	3.4	3.0
	a	0.7	0.5	0.6
	b	0.9	0.5	0.6
	c	1.1	1.2	1.1
Mucoprotein —N $\times$ 12.5		0.29	0.18	0.34

\* The material designated  $\alpha_2$ -globulin is an abnormal component migrating between the  $\alpha_1$ - and  $\alpha_2$ -globulin.

*Electrophoretic Isolation of M-1.* Since the M-1 component becomes well separated from other components of serum at pH 4.5, it is well suited to electrophoretic separation. An experiment was carried out in which serum was undiluted, except for the dilution taking place during dialysis, and in which electrophoresis was carried out in an 11 ml analytical cell with 2 center sections. Electrophoresis was carried out, with compensation, until the ascending boundary of the M-1 component had nearly reached the top of the cell, and the ascending M-2 boundary was below the middle junction of the cells. One half of one channel of the cell could then be isolated, containing M-1 alone. This material was subjected to chemical analysis, with the results given in Table III. There can be no doubt that the material represented by the M-1 peak at pH 4.5 has the same general chemical characteristics as the mucoprotein isolated from perchloric acid filtrates of serum or plasma.

*Discussion.* The identity of the M-1 component of the electrophoresis pattern of serum at pH 4.5 and the fraction of the isolated mucoprotein which we have designated as MP-1 seems to be adequately established by the experiments presented. The addition of the isolated material to serum has confirmed the identity of the electrophoretic mobilities,

TABLE III.  
Analytical Values for Electrophoretically Isolated M-1.

Tyrosine	7.6 mg%
Carbohydrate	34.4 "
CHO/T	4.5
Nitrogen	16.2 mg%
Glucosamine	28.5 "
CHO/G	1.2

\* These refer to concentration in the solution removed from the cell.

and the material isolated by electrophoresis has been shown to have chemical properties similar to the material isolated by fractional precipitation.

The nature of the M-2 component of the electrophoresis pattern of serum at pH 4.5 is not established. The mobility does not correspond entirely with that of any component of the isolated mucoprotein. The increase in the M-2 component resulting from the addition of isolated mucoprotein containing fractions other than MP-1 would suggest that M-2 is also a mucoprotein. The association of increases in M-1 and M-2, and the rather low isoelectric point of M-2 would point in the same direction, but certainly without being conclusive. The solution of the problem will require the isolation of a homogeneous preparation which has the electrophoretic properties of M-2 by itself or on addition to serum. The isolation of such material is now being investigated.

TABLE I.

Analytical Values Obtained on Perchloric Acid Filtrates from the Sera of 3 Patients, GAM, BUR, and HUM. Concentrations are expressed as mg per 100 cc of original serum, and were obtained by the methods previously described.<sup>1</sup> Average values of the ratios of carbohydrate:tyrosine (CHO/T) and carbohydrate:glucosamine (CHO/G) obtained on mucoprotein isolated from normal human plasma<sup>1</sup> are given for comparison.

Sample	Tyrosine, mg%	CHO, mg%	CHO/T	N, mg%	Protein (Biuret) mg%	Glucos- amine, mg%	CHO/G
GAM carcinoma	9.7	52.0	5.3	23.5	240.0	33.5	1.55
BUR (pneumonia)	10.8	55.0	5.1	27.5	270.0	33.5	1.64
HUM (pneumonia)	5.7	27.5	4.8	14.5	—	21.0	1.31
Normal	—	—	3.6	—	—	—	1.27

scribed<sup>1</sup> and contained in addition to MP-1 the slower components (MP-2 and MP-3) seen in these preparations.<sup>3</sup> The addition of such material to serum is seen to result in an increase in both the M-1 and M-2 fractions of the resulting mixture. The mobility of the M-1 fraction in serum corresponds closely with that of the MP-1 component of isolated mucoprotein. The mobility of the M-2 fraction of serum, however, ( $-1.4$  to  $-2.0 \times 10^{-5}$ ) does not agree with the mobility of either the MP-2 ( $-2.4$  to  $-2.8 \times 10^{-5}$ ) or MP-3 ( $-0.5$  to  $-1.2 \times 10^{-5}$ ) of the isolated preparations. When such preparations are added to serum, we have been able to find satisfactory correspondence only between the mobilities of the M-1 and MP-1 fractions.

*The relation of the electrophoretic components M-1 and M-2 to chemically determined mucoprotein.* Electrophoresis patterns of a number of pathological sera at pH 4.5 are shown in Fig. 3. It will be seen that an increase in M-2 is found to accompany an increase in M-1 in either carcinoma or pneumonia. In each instance, the level of mucoprotein-tyrosine (MPT) in mg percent in the original plasma<sup>2</sup> is also indicated. The mucoprotein-tyrosine is between 9 and 11 mg percent in all cases except that of BAR, where it is 4.5. Inspection of the electrophoresis patterns will make it evident that the area of the M-1 peak is about the same in all except that of BAR, and that there is at least a rough correspondence between the amount of M-1 and the MPT. The amount of M-2, however, does not seem to bear any quantitative relationship to the amount of MPT.

Serum samples from three patients were studied in somewhat greater detail. Sufficient

serum was obtained in each case to carry out the electrophoresis at pH 8.4 and pH 4.5; to prepare perchloric acid filtrates for the determination of the mucoprotein-tyrosine, as well as the carbohydrate, nitrogen, glucosamine, and the value of the biuret reaction.<sup>1</sup> The results of the chemical analyses of the filtrates are given in Table I, together with the ratios of carbohydrate:tyrosine and of carbohydrate:glucosamine for these filtrates and for the mucoprotein isolated from normal plasma. In Table II the amount of each electrophoretic component is calculated and compared with the amount of mucoprotein indicated by the chemical determination. This calculation is based upon a nitrogen content of 7.9%, determined upon the isolated mucoprotein. It must be recognized, of course, that the accuracy with which the areas of the small electrophoretic peaks for M-1 can be determined is not great, and that the translation of these areas into concentrations involves the assumption that the mucoprotein would have the same refractive index increment as that of the other serum proteins. It seems reasonable to conclude, however, that the agreement between the electrophoretic and chemical values indicates that the chemical determination is primarily a measure of M-1. Certainly, the sum of M-1 and M-2 is too great to be accounted for by the material in the perchloric acid filtrates.

In comparing the results of the electrophoresis experiments at pH 8.4 and 4.5, it is seen that M-1 does not exceed  $\alpha_1$ -globulin in any of these cases, but may be considerably smaller (as in the case of HUM). On the other hand, the amount of M-1 plus M-2 may be considerably larger than the amount of  $\alpha_1$ -globulin.

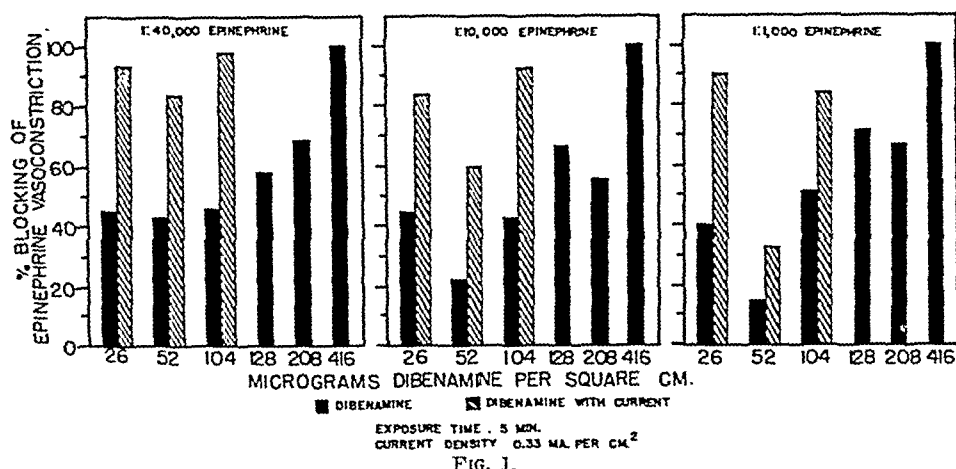


FIG. 1.

Effects of ion transfer on the cutaneous adrenergic blocking activity of Dibenamine.

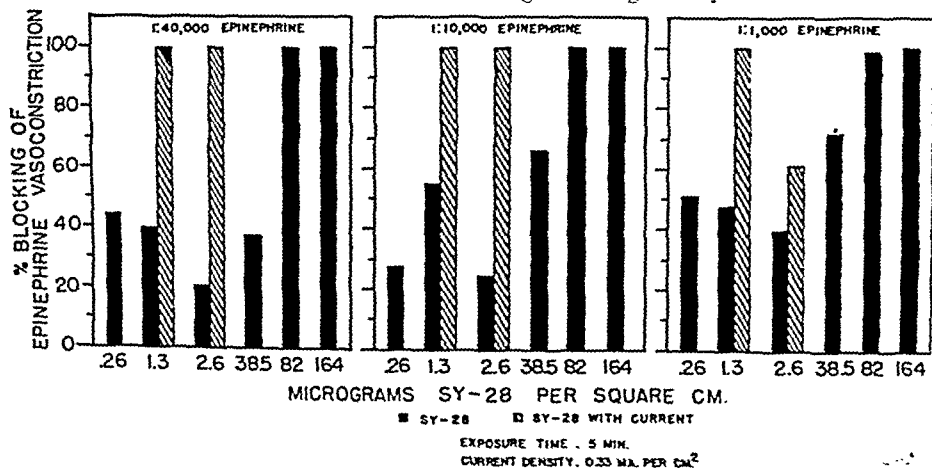


FIG. 2.

Effects of ion transfer on the cutaneous adrenergic blocking activity of SY-28.

imum in 30 minutes and manifested itself by a circular area of blanching.

The diameters of the blanched skin were measured and the areas computed. These areas were then weighted for intensity of vasoconstriction. This was accomplished by arbitrarily recognizing four degrees of response and assigning an intensity number for each degree. Full, faint, very faint, and no vasoconstriction, (*i.e.* complete blocking), were assigned the numbers 3, 2, 1 and 0 respectively. The area multiplied by the intensity factor yielded a weighted area of vasoconstriction. The percentage of blocking was then easily obtained mathematically by comparing the treated sites with the control sites.

The results were analyzed statistically by the method of paired comparisons. After calculating the statistic "t", the probability value "P" was obtained from Fisher's "t" table.

**Results.** The data obtained for Dibenamine and SY-28 are illustrated in Fig. 1 and 2. Since the absolute amount of drug present in the skin following exposure was unknown, the values plotted along the abscissae were the amounts actually applied to the skin.

Dibenamine produced complete blocking of epinephrine at a dose of 416 μg per sq cm whereas SY-28 produced the same effect with 82 μg per sq cm. Under these conditions SY-28 was approximately 5 times as potent as Dibenamine in blocking epinephrine. The ap-

**Summary.** When serum is subjected to electrophoresis at pH 4.5, two components are seen which have isoelectric points more acid than those of albumin. One of these has been identified with a mucoprotein which has been shown to increase in amount in cancer, pneu-

monia, and certain other diseases. The second component, with a less acid isoelectric point, may also be a mucoprotein, but this point has not been definitely established.

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## Effect of Adrenergic Blocking Agents on the Cutaneous Action of Epinephrine. (17349)

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Adrenergic blocking drugs are of clinical interest in the treatment of conditions characterized by the element of vasospasm, such as early Buerger's disease, Raynaud's disease and causalgic states. It was felt that by the direct diffusion or by ion transfer through the skin, it would be possible to avoid the generalized toxic manifestations encountered by way of the parenteral route.<sup>1</sup> The adrenergic blocking agents selected for this study were N-(2-chloroethyl) dibenzylamine hydrochloride (Dibenamine) introduced by Nickerson and Goodman<sup>2</sup> and N-(2-bromoethyl)-N-1-naphthalenemethylamine hydrobromide (SY-28) introduced by Loew and Micetich.<sup>3</sup> Prior to application in the human, data were obtained in a group of albino rabbits.

**Methods.** Albino rabbits weighing 2.0 to 4.5 kg were used. The abdomen and thorax of the animals were depilated with a special sulfide-detergent mixture<sup>4</sup> 24 hours prior to use. Solutions of Dibenamine and SY-28 in propylene glycol were freshly prepared prior to each experiment. Four-tenths ml of these solutions or of solvent controls were applied to rectangular strips of asbestos paper 1.25

by 12.5 cm in size. This amount of solution was sufficient to saturate the pads. The strips were then applied to the abdomen in 4 parallel sites in the following order: solvent without current, solution containing the drug without current, solution containing the drug plus current, solvent plus current. Two to four rabbits were used at each of the various concentrations of blocking agents studied. Since the active portions of both Dibenamine and SY-28 are cationic, the anode of a suitable direct current source\* was used to transfer the agent into the skin. A current density of 0.33 ma/cm<sup>2</sup> or a total current of 5.2 ma was employed. This dosage of current was arbitrarily selected as being just below the pain threshold for human skin and was well tolerated by the rabbits. The solutions were placed in contact with the skin for exactly 5 minutes with or without current. Immediately following the 5 minute application, the asbestos pads were removed and the blocking agent wiped off with propylene glycol.

To measure the adrenergic blocking activity of the treated sites, 0.1 ml of 1:1,000, 1:10,000 and 1:40,000 epinephrine solutions were injected intracutaneously 30 minutes following application of the blocking agents. An equal amount of physiological saline served as a control. The injection sites were randomized. Vasoconstriction reached a max-

<sup>1</sup> Hecht, H. H., and Anderson, R. B., *Am. J. Med.*, 1947, 3, 3.

<sup>2</sup> Nickerson, M., and Goodman, L. S., *Proc. Am. Fed. Clin. Research*, 1945, 2, 109.

<sup>3</sup> Loew, E. R., and Micetich, A., *Fed. Proc.*, 1947, 6, 304.

<sup>4</sup> Pitesky, I., and Last, J. H., *Science*, 1948, 108, 657.

\* A McIntosh Wall Plate was used. This machine is manufactured by the McIntosh Electrical Corp., Chicago, Ill.

## Stripping Film Technic for Radioautographs. (17350)

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(Introduced by E. M. Landis.)

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The method of radioautography for localization of radioactive tracers in tissues is finding increasingly broad application as a new approach to histochemistry. One of the potential hazards in connection with all histochemical technics, including that of radioautography, is the leaching out or displacement of material under investigation which may occur as a result of tissue processing or staining. For example, studies at this laboratory<sup>1</sup> on radioautographic localization of  $P^{32}$  in brain tissue have shown that marked losses of the tagged element are incurred in the course of fixing and staining for routine paraffin sections. Perhaps the surest way to avoid artifacts of this nature is to use completely unprocessed frozen material for the radioautograph exposure, omitting all fixing and staining until after the autograph has been completed. An advantage of the method to be described is that it not only makes such post-staining possible, but also makes the staining process quite independent of the radioautograph development. As a result, the tissue may be stained by any of the well-documented histochemical technics as adjunct to or control of the isotope localization. An outline of the technic and an example of its application has been described in a preliminary report from this laboratory.<sup>2</sup> Similar applications of stripping film have also been reported

by Pelc, Boyd and others.<sup>3</sup> A more general discussion of radioautographic technics may be found in the review article by Evans<sup>4</sup> and the book of Yagoda.<sup>5</sup>

**Method. A. Films.** In a series of experiments<sup>6</sup> in which nineteen commercial emulsions were investigated with respect to sensitivity, contrast and grain size, Type M stripping film (now commercially available) was selected as best suited for our radioautograph technic on the basis of the following qualities: fine grain, high contrast, low background fog and thinness of emulsion. Emulsions with finer grain and consequently greater resolving power are available (as, for example, Ansco Reprolith ortho stripping film), but these are very much less sensitive and so call for exposures which are impracticable for most work. Type M stripping film represents a satisfactory compromise between the mutually exclusive extremes of fine grain and high sensitivity in commercial emulsions.

The emulsion of Type M stripping film is about 10 microns in thickness and is coated on a 7 micron transparent cellulose acetate stripping base. Emulsion and stripping base are in turn mounted on a transparent supporting base about 0.15 mm thick, as shown in Fig. 1. At the time of use the emulsion on its 7 micron base is stripped away from the supporting material and can then be handled almost as though it were an autonomous 10 micron layer of emulsion. To facilitate later steps, it is advantageous to have on hand pieces of film just large enough to overlap the edges of a standard microscopic slide.

**B. Tissue Preparation.** 1. Frozen sections. Our best histology has been obtained by freeze-

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<sup>1</sup> Steinberg, D., and Selverstone, B., unpublished data.

<sup>2</sup> MacDonald, A. M., Cobb, Jock, and Solomon, A. K., *Science*, 1948, **107**, 550.

<sup>3</sup> Pelc, S. R., *Nature*, 1947, **160**, 749; Boyd, G. A., and Williams, A. I., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 225; Endicott, K. M., and Clark, F. A. (Ref. 5, p. 48).

<sup>4</sup> Evans, T. C., *Nucleonics*, 1948, **2**, 52.

<sup>5</sup> Yagoda, H., *Radioactive Measurements with Nuclear Emulsions*, John Wiley and Sons, Inc., New York, 1949.

<sup>6</sup> Cobb, Jock, and Solomon, A. K., *Tex. Sci. Inst.*, 1948, **10**, 441.

plication of current enhanced the penetration of both drugs in a statistically significant manner. Analysis of the epinephrine responses in 4 rabbits following the use of Dibenamine in a concentration of  $104 \mu\text{g}/\text{cm}^2$  with and without ion transfer yielded the following "P" values at various epinephrine concentrations:

- a. 1:1,000 -  $P = 0.01$
- b. 1:10,000 -  $P = 0.06$
- c. 1:40,000 -  $P = 0.04$

Correspondingly, with SY-28 in 4 rabbits with and without ion transfer at a dosage of  $1.3 \mu\text{g}/\text{cm}^2$  the following P values were calculated for the concentrations of epinephrine used:

- a. 1:1,000 -  $P = 0.04$
- b. 1:10,000 -  $P = 0.07$
- c. 1:40,000 -  $P = <0.01$

Dibenamine and SY-28 were found to be fixed locally, since the skin surrounding the treated site responded to injected epinephrine in a normal manner. During the course of the study, it was noticed that an area of erythema developed in the sites injected with epinephrine prior to the appearance of vasoconstriction. This response was even more marked and persistent when vasoconstriction was completely blocked. The process of ion transfer produced minimal erythema which made it difficult to evaluate the subsequent erythematous response to epinephrine. For this reason two groups of 8 rabbits each were given Dibenamine or SY-28 by the intravenous route. Epinephrine was injected intracutaneously as in the previous experiments 30 minutes after the injection of the blocking drug. Erythema developed rapidly and either disappeared over a 15 minute period or was followed by vasoconstriction in the situations where incomplete blocking occurred. This "epinephrine reversal" type of phenomenon suggests the presence of vasodilator fibers in the skin of the rabbit.

Fig. 3 and 4 illustrate a dose response relationship when the blocking agents were given intravenously. One hundred percent blocking of epinephrine vasoconstriction occurred when 32 mg of Dibenamine per kilo were given. The same effect was achieved with SY-28 when 4 mg per kilo were injected. Thus

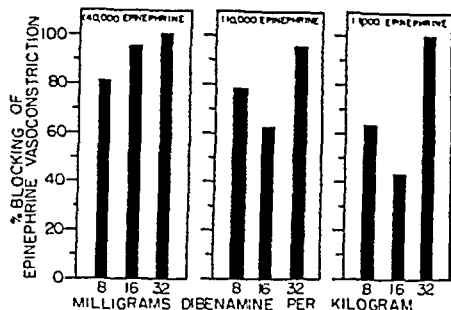


FIG. 3.  
Cutaneous adrenergic blocking activity of intravenous Dibenamine.

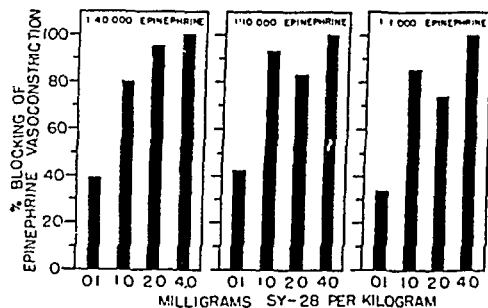


FIG. 4.  
Cutaneous adrenergic blocking activity of intravenous SY-28.

SY-28 was approximately 8 times as potent as Dibenamine when given by the intravenous route.

**Summary.** 1) Both Dibenamine and SY-28 were absorbed by the intact rabbit skin and were capable of blocking the vasoconstriction produced by intracutaneously administered epinephrine. By this route SY-28 was approximately 5 times as potent as Dibenamine.

2) When administered by ion transfer, the penetration of both drugs into the skin was significantly facilitated.

3) Both blocking compounds were fixed locally in the skin. Contiguous untreated areas were still capable of responding to epinephrine.

4) By the intravenous route, SY-28 was approximately 8 times as potent as Dibenamine.

5) The "epinephrine reversal" phenomenon has been noted for the first time in the skin of the rabbit and suggests the presence of vasodilator fibers.

parent supporting base is laid over the emulsion to serve as protection, and the preparation is smoothed with the fingers. Finally, a second microscope slide is placed on top of the supporting base and the entire "sandwich" wrapped with scotch tape. It is our practice to wrap each preparation individually in opaque paper and place it immediately under a pressure of 8 lbs/sq inch. Resolution is considerably improved by the more intimate tissue-film contact obtained with pressure. After 24 hours under weights the preparation is transferred to the refrigerating unit or dry ice box and stored at freezing temperature for the duration of the exposure time. Removal from under the weights at this time, after the celloidin has set, causes no apparent loss in resolution. Estimation of proper exposure times will be discussed below.

D. Developing and Mounting. Autographs ready for development are unwrapped in the darkroom and the scotch tape is cut along the edges of the "sandwich" with a razor blade. Unwinding the tape leads to a grossly visible blue static discharge which can fog the film quite badly. After removal of the guard slide and the supporting base, the bottom slide, bearing tissue and adherent stripping film, is placed in the developer *in toto*. It is carried as a unit through the stop bath and fixative. Each slide is developed in a separate test tube into which the developer and other solutions are successively poured.

In order to control the many variables that may affect film sensitivity we have adopted the following precautions as part of our standard procedure. (a) All films are kept in a desiccated box in the refrigerating unit. (b) Films are allowed to come to room temperature before use. (c) Exposures are all made at the same temperatures. (d) Films are developed immediately at the end of the exposure period. (e) Wratten Series 2 safelight at 3 feet is the only illumination used.

The developing process is controlled in the following ways. (a) Distilled water is used in all solutions. (b) Developer is stored in full, brown, stoppered bottles. (c) 50 cc of fresh developer is used for each preparation. (d) Each tube is inverted gently every 30

seconds during development. (e) All solutions are brought to  $68^{\circ} \pm 0.5^{\circ}\text{C}$  and kept at this temperature during development.

During the processing, the emulsion is directly and evenly exposed to the solutions, thus insuring complete, uniform development. The tissue is protected by the intervening stripping base and the celloidin coating, so that it is preserved from the action of the developing solution. Since the stripping film gradually separates from the slide during processing, however, it is important to limit the time in the solutions. Five minutes in Kodak D-19 developer, 15 seconds in 1% acetic acid stop bath and two minutes in Kodak F-5 fixing-hardening solution constitutes a satisfactory routine procedure. After 5 to 10 minutes washing in cold water, the stripping film has become partially detached from the slide and a gentle, steady pull on one end suffices to complete the separation. The tissue section adheres to and comes away with the stripping film which can be left in a beaker of distilled water until it is mounted.

At this point we have the tissue section adherent to one side of the stripping base and on the opposite side the developed grains of the tissue's own radioautograph. We now simply take this preparation up through alcohols to xylol, and, after trimming away the excess film around the tissue, mount with balsam, or a substitute, and a cover slip. Usually, it does not matter whether the tissue is above or below the autograph, but when the image is very dense, the tissue detail is better examined with the tissue uppermost. The orientation of the final preparation is shown in Fig. 3.

At low magnification, the close juxtaposition of tissue with its autograph makes it possible to survey the preparation with a single fixed plane of focus. At high magnification, a slight change of microscope focus correlates the grain density pattern in the autograph with the histologic pattern.

E. Post-staining. When unstained sections have been used, the steps are exactly the same up to the point at which the preparation is removed from the wash water. In order to stain the tissue and at the same time to





FIG. 1.

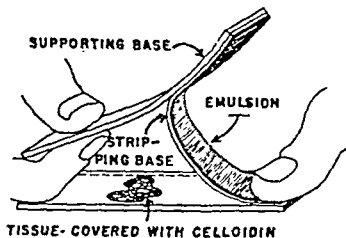


FIG. 2.

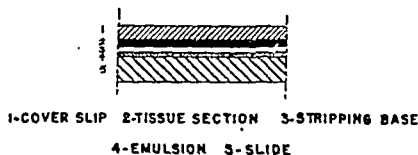


FIG. 3.

ing the tissue to be studied immediately on removal in iso-pentane at liquid nitrogen temperatures, according to the technic of Linderstrøm-Lang and Mogensen.<sup>7</sup> It is then transferred to a refrigerated room at  $-10$  to  $-15^{\circ}\text{C}$  and cut on a microtome equilibrated at that temperature, making use of a modified Linderstrøm-Lang "window",<sup>8</sup> to prevent rolling of the sections. These are melted onto slides previously prepared by coating with 0.1% gelatin which is then hardened by dipping in 10% formalin. With this technic sections can be allowed to dry at room temperature without distortion. They are then ready for the application of the film in the manner described below.

2. Paraffin sections. When there is no problem of leaching out or displacement of the material under investigation, routine fixing, embedding and sectioning technics are employed. Needless to say, tissue detail obtained with this technic is decidedly superior

to that of even the best frozen sections, and hence this technic is to be preferred whenever possible. The slides are stained in the usual manner up to the absolute alcohol stage. They are not carried through into xylol since the film is to be mounted with an alcohol base adhesive mixture. If the film cannot be mounted at the time staining is completed, the slides may be carried through to xylol, provided it is removed with absolute alcohol prior to mounting.

C. Application of the Film. All handling of film is carried out under Wratten Series 2 safelight. First, the emulsion and stripping base are separated from the heavy supporting base as shown in Fig. 1, leaving the last  $\frac{1}{4}$  inch adhering to the base to facilitate manipulation. Stripping can usually be started at a corner of the film by stroking the emulsion away from the support with the ball of the thumb.<sup>9</sup> Stripping should be done slowly to prevent any static discharge between stripping base and support, as such a discharge causes a considerable increase in background fog.

A 1% solution of celloidin in methyl alcohol is used to cement the film, *emulsion side up*, to the tissue. If the celloidin is dissolved in the conventional ether-alcohol mixture, irregular black splotches appear on the developed film. These artifacts have been eliminated by the use of methyl alcohol as solvent. The slides are laid tissue section up on a piece of filter paper and the alcohol from the paraffin sections is allowed to evaporate almost completely. Then a drop or two of the celloidin solution is placed directly on the tissue section and another drop on the slide to either side of the section. The partially stripped film is then applied to the section as shown in Fig. 2, and stripping of the film is completed at the same time. Next, the trans-

<sup>9</sup> To facilitate stripping, Dr. George A. Boyd<sup>13</sup> has suggested turning the film emulsion side down and slitting the supporting base with a razor blade. The backing can then be cracked along the slit. While this procedure simplifies stripping, it also increases the background fog due to the static discharge accompanying the breaking of the support. See Boyd's article for details.

<sup>7</sup> Linderstrøm-Lang, K., and Mogensen, K. R., *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, 1938, 23, 27.

<sup>8</sup> Coons, A. H., private communication.

TABLE I.  
Initial Activity Required in Disintegrations per Minute per cm<sup>2</sup> of Tissue.

	Eastman no-screen x-ray film		Eastman type M stripping film	
	0.1 > Bkgd.	0.6 > Bkgd.	0.1 > Bkgd.	0.6 > Bkgd.
Ci <sup>114</sup>	124	806	2,280	11,400
Ca <sup>45</sup>	124	1,030	3,260	27,000
I <sup>131</sup>	414	2,180	18,500	75,200
P <sup>32</sup>	843	4,610	36,700	160,000
Zn <sup>65</sup>	4,250	27,400	138,000	741,000

Values given assume a 15-day exposure and 5 minutes development in Kodak D-19. Data are for a uniform source, and so apply only to mean activity of tissue section.

ticular structure and so the average density of the autograph may be a very unreliable guide to optimum exposure. Both when counting the initial activity of tissue sections and when examining the images of preliminary No-Screen x-ray autographs, these areas of interest should where possible be the basis of estimation. It is absolutely essential that the precautions in handling of films and development described above be observed in order to obtain reproducible results.

*Discussion.* The advantages of the present technics are several:

1. The staining and developing processes are independent of one another.
2. The thickness of the emulsion is equal over the entire preparation so that differences in grain density at different points can be used as an index of the activity of the underlying point in the tissue.
3. Tissue and emulsion are held in a fixed relationship from the very beginning of the procedure through to the time of examination. Since one can look through both the autograph and the tissue simultaneously under the microscope, correlation and interpretation are made simple.

One limitation of the technic lies in the 7 micron separation of tissue and emulsion. While in many studies this very small separation may not be of importance, it does limit the theoretical resolution possible with the technic. However, our results with Type M stripping film indicate that resolution to 10-15 microns can be obtained, as illustrated by Fig. 4 and 5.

These autographs show the very different rates of uptake of I<sup>131</sup> by different thyroid follicles. The fine grain of the emulsion can

be noted and the order of magnitude of the background can be estimated from the areas of emulsion not overlying tissue. At 16 hours after an injection of iodide the material is known to be widely disseminated throughout the gland and this is evidenced in the autographs. Of particular interest is the rather sharp demarcation of the high activity colloid material from the low activity thyroid stroma.

The data of Table I for Type M stripping film should make clear a limitation of the radioautographic technic that is too seldom emphasized, the need for high tissue activity. Unless the material under investigation is highly concentrated by the organism as in the case, say of radioiodine, the specific activity requirements are high and in some cases forbiddingly high. With the aid of data on exposure, such as that referred to above, it is often possible to determine beforehand the feasibility of a study when the specific activity of the material and the probable biologic dilution are known.

*Summary.* A technic for radioautography is described which makes the developing and staining processes independent and, through the use of stripping film, provides an emulsion of uniform thickness. The method of mounting permits uniform development of the entire emulsion and maintains a fixed tissue-autograph relationship.

We should like to express our thanks to Dr. A. H. Coons for advice and assistance in the technic of cutting frozen sections. One of us (A.M.M.) has held a Rockefeller Fellowship in Pathology. This work has been supported in part by the Office of Naval Research and the Atomic Energy Commission.

protect the emulsion from the stain the stripping film is now cemented *emulsion side down* onto a clean slide with Kodalith Stripping Film Cement or with 1% celloidin in ether-alcohol. This leaves the tissue section exposed and the slide can be handled through the staining process in the routine manner. After staining, the excess film peripheral to the tissue section itself is trimmed away and the preparation covered with balsam, or a substitute, and a cover slip. In order to obtain a uniformly flat field it is necessary to weight the cover slip rather heavily until the balsam has dried.

**F. Time of Exposure.** Data have been presented elsewhere<sup>9</sup> that permit the calculation of exposure time for Type M stripping film for 5 important isotopes,  $C^{14}$ ,  $Ca^{45}$ ,  $I^{131}$ ,  $P^{32}$ , and  $Zn^{65}$ , provided the activity of the preparation in terms of disintegration per minute per unit area is known. For problems in which such assay is inconvenient, a satisfactory empirical procedure has been developed, based on the use of Eastman No-Screen x-ray film, a film many times more sensitive than the Type M. Using this film, "trial" radioautograph preparations are made using tissue sections from the same block. The film is merely laid over the tissue section without adhesive, covered with a guard slide and "sandwiched" with scotch tape as above. At the time of development the "sandwich" is opened and the film removed and processed. Several such preparations are put up and developed at intervals. From the ratio of sensitivities of the No-Screen x-ray and Type M stripping films the exposure time needed to get an equivalent density with the latter can be calculated. Ratios of initial activity requirements from Table I indicate the relative sensitivities of the two films.

Table I (taken from reference 9) gives the activities required at the surface of the tissue to produce radioautographs of image density 0.1 and 0.6 above background with 15 day exposures to uniform sources of  $C^{14}$ ,  $Ca^{45}$ ,  $P^{32}$ ,  $I^{131}$  or  $Zn^{65}$ . It must be kept in mind that generally one is interested in some par-



Fig. 4.

Radioautograph of rabbit thyroid. Animal was sacrificed 16 hours after intraperitoneal tracer dose of 270 microcuries  $I^{131}$ . Tissue was formalin fixed and stained with hematoxylin-eosin. Type M stripping film, exposed 16 hours, developed 5 minutes in Kodak D-19. Magnification 200 $\times$ .



Fig. 5.

Enlargement of area blocked out in Fig. 4. Upper: Focused in plane of tissue to bring out histologic detail. Lower: Same area focused in plane of photographic grains for study of distribution and contrast. Magnification 800 $\times$ .

<sup>9</sup>Steinberg, D., and Solomon, A. K., *Rev. Sci. Inst.*, in press.

TABLE I.  
Lack of Protective Effect of Phenergan Against Acute Pulmonary Edema Produced by Epinephrine or Ammonium Chloride.

Exp. No.	Treatment	Avg body wt, g	Dose of phenergan, mg/kg	Mortality ratio*	Median survival time,† min.	Avg lung wt,‡ g/kg body wt
Rats						
I	NH <sub>4</sub> Cl	199	—	8/15	35	11.6
II	" + phenergan	220	20	4/10	20	18.2
III	" + "	206	40	8/10	24	14.6
IV	Phenergan	196	40	0/10	—	6.2
V	No drug	212	—	0/4	—	7.7
Guinea pigs						
VI	NH <sub>4</sub> Cl	461	—	8/10	8	10.4
VII	" + phenergan	408	20	12/12	8	8.6
VIII	Epinephrine	436	—	6/7	7	19.7
IX	Epinephrine + phenergan	423	20	8/8	7	20.3
X	No drug	450	—	0/5	—	6.8

\* Mortality ratio = number dead/number used.

† Excluding those surviving indefinitely.

‡ Avg lung wt in experimental groups include only those animals which succumbed after injection. Control animals were sacrificed under pentobarbital anesthesia.

time of the animals which died. The difference in lung weight between Groups VI and VII is not significant ( $P > 0.05$ ), but Group VII differs significantly from control Group X ( $P = 0.02$ ), as does Group VI ( $P < 0.01$ ). In all the other cases, the differences between experimental and control groups are obvious from inspection of the table.

**Discussion.** The lung weights of the guinea pigs succumbing to epinephrine were greater than in those receiving a lethal dose of ammonium chloride. It may therefore be assumed that pulmonary edema was not maximal in the latter group, and may not have been the sole cause of death. Stone and Loew<sup>7</sup> have also expressed the view that pulmonary edema may not have been the sole cause of death in their epinephrine treated rabbits. The overall mortality rate in all our animals receiving edema-producing drug without Phenergan was 22/32 or 69%, while in those receiving Phenergan in addition to the toxic agent it was 32/40 or 80%. Our results are in agreement with the statement of Stone and Loew,<sup>7</sup> that Phenergan did not protect the animals against pulmonary edema, although we did not observe the lower mortality rate which they reported in their animals.

Koenig and Koenig<sup>8</sup> believe that the pulmonary edema produced by ammonium chlo-

ride is a specific action of the ammonium moiety, and is not related to the acidosis produced by NH<sub>4</sub>Cl. Other ammonium salts also induced pulmonary edema, while acidosis produced by other means did not. However, they did not publish data indicating the degree of acidosis produced by the various agents they used, and it is therefore impossible to judge the validity of their conclusions.

The divergence between our results and those of Reuse<sup>3</sup> is not easy to explain. He used guinea pigs about twice as large as those herein reported. He also used epinephrine ascorbate, while the epinephrine we used was Adrenalin chloride (Parke, Davis), a glandular extract containing an unknown amount of nor-epinephrine. Halpern<sup>1</sup> stated that Phenergan did not prevent the blood pressure and cardiac effects of epinephrine, but Reuse<sup>7</sup> found that Phenergan in large doses inhibited both the pressor effect and the apnea produced by small doses of epinephrine. This effect, however, lasted only a few minutes, and probably does not explain the partial protection which he observed. Even in Reuse's experiments, Phenergan did not offer complete protection against epinephrine-induced pulmonary edema, as the

<sup>7</sup> Reuse, J., *Ann. soc. roy. d. sciences med. et nat. d. Bruxelles*, t. II, 1949.

# Failure of Antihistaminic Drug "Phenergan" to Protect Against Acute Pulmonary Edema. (17351)

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Halpern and his co-workers have developed the thesis that antihistaminic drugs owe much of their therapeutic effectiveness to a primary influence upon cellular permeability, aside from their property of competitive inhibition of histamine. This concept is based in part on the observation that N-( $\beta$ -dimethylamino- $\alpha$ -methylethyl)-phenothiazine (3277 R.P. or Phenergan) protects guinea pigs and rabbits from pulmonary edema produced by an overdose of epinephrine<sup>1,2</sup> or by certain poison gases, such as chloropicrin.<sup>3</sup>

On the other hand, Stone and Loew<sup>4</sup> failed to obtain protection by pyranisamine (Neo-Antergan) in rabbits with epinephrine-induced pulmonary edema. The same authors<sup>5</sup> also could not confirm the observation that Phenergan protected rabbits. In one series (8 animals) they obtained a reduced mortality but no reduction in pulmonary edema, as judged by lung weight. It seemed of interest to determine whether Halpern's results could be confirmed in our laboratory, and also whether Phenergan would affect pulmonary edema produced by administration of ammonium salts, as described by Koenig and Koenig.<sup>6</sup> If it could be shown that pulmonary edema produced by two such widely different means could be alleviated by an antihistaminic drug, support would be added to

Halpern's view that such drugs may primarily affect cellular permeability.

**Methods.** The rats used in this study weighed about 200 g. Both Carworth and Holtzmann rats were employed, and as no difference was noted in results obtained with the two strains, they have been combined in the summary. The guinea pigs varied from 300 to 500 g in weight. Pulmonary edema was produced as follows: epinephrine chloride was injected in the forelimb vein of guinea pigs, 0.6 mg per kg (the dose used by Reuse<sup>3</sup>); ammonium chloride was administered intraperitoneally, the dose being 400 mg per kg for rats, and 600 mg per kg for guinea pigs, as described by Koenig and Koenig.<sup>6</sup>

Phenergan was chosen as the antihistaminic drug, because Halpern has stated that its effect on cellular permeability was greater than any others he used. We administered Phenergan subcutaneously, except to a few of the rats, where the intraperitoneal route was employed. The dose was the same as that used by Halpern and by Reuse; namely, 20 mg per kg, except in 2 groups of rats where the dose was doubled. Thirty to 60 minutes (usually the latter) were allowed to elapse between injection of Phenergan and of epinephrine or ammonium chloride. The lot of Phenergan used was manufactured in France, and obtained from Société des Usines Chimiques Rhone-Poulenc, Paris.

**Results.** The syndrome of acute experimental pulmonary edema in rats and guinea pigs has been adequately described by Koenig and Koenig.<sup>6</sup> The signs and gross findings at autopsy described by them were seen in the majority of our animals.

The results are summarized in Table I. It is evident that Phenergan, even in the relatively large doses used, did not protect the animals from pulmonary edema as judged by lung weight, nor did the antihistaminic drug reduce the mortality, nor prolong the survival

<sup>1</sup> Halpern, B. N., Hamburger, J., and Cruchaud, S., *Acta allerg.*, 1948, **1**, 97; Halpern, B. N., and Cruchaud, S., *Compt. rend. soc. biol.*, 1947, **141**, 1038; Vermeil, G., Halpern, B. N., and Cruchaud, S., *Compt. rend. soc. biol.*, 1949, **143**, 77.

<sup>2</sup> Reuse, J., *Compt. rend. soc. biol.*, 1948, **142**, 638.

<sup>3</sup> Halpern, B. N., and Cruchaud, S., *Compt. rend. Acad. d. sc.*, 1947, **225**, 1194.

<sup>4</sup> Stone, C. A., and Loew, E. R., *Fed. Proc.*, 1949, **8**, 335.

<sup>5</sup> Stone, C. A., and Loew, E. R., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 122.

<sup>6</sup> Koenig, H., and Koenig, R., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 375.

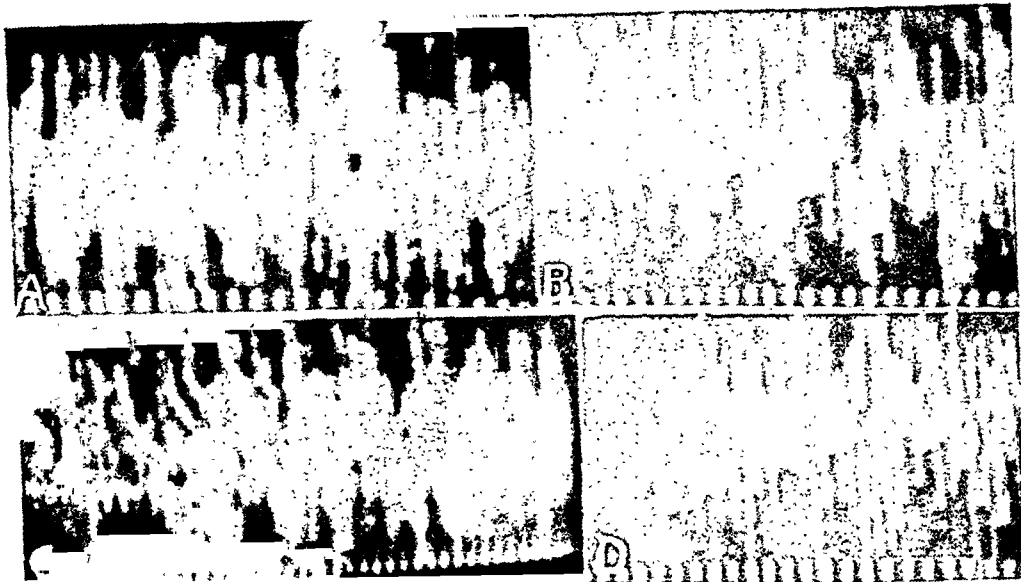


FIG. 1.

Electrical activity of the cerebellum recorded from the pial surface with bipolar silver wire pick-up electrodes. 100/sec. time signal.

A. Cat under nembutal anesthesia. Five days previously all 6 cerebellar peduncles had been transected, thus interrupting all afferent and efferent fibers. Note normal activity.

B. Cat under nembutal anesthesia. Note electrical activity in cerebellar cortex dissected free of animal and lying on saline moistened gauze. Nuclear cells were not present.

C. Cat under nembutal anesthesia. Note "normal" electrical activity, despite the total bilateral destruction of cerebellar nuclei.

D. Cat under nembutal anesthesia. Note electrical activity similar to A, B, C, above. Cerebellar peduncles and cerebellar nuclei left intact.

in Fig. 1b which shows the electrical activity in a part of the cerebellum which was dissected free and was lying on a strip of gauze several centimeters from the animal. Such activity, however, does not continue for more than 2-4 minutes

Since the fast electrical activity can continue in the absence of all fiber connections with the rest of the nervous system, then it must be maintained by intrinsic mechanism within the cerebellum.

The next question was: What are the intrinsic mechanisms which maintain this electrical activity? The cerebellar cortex is an ideal preparation with which to work because the anatomy is simple, well known, and remarkably uniform in structure. Fig. 2 summarizes the present state of our information on the anatomy of this organ. Working on the assumption that this activity is maintained either by reverberating circuits, or spontaneous activity of cellular aggregates, or both,

the following analysis has been made:

*Reverberating Circuits.* A glance at Fig. 2 indicates the possibility of two reverberating circuits: 1. Purkinje cell (A) to central cerebellar nuclei, (B) back to Purkinje cell. That this circuit cannot be the responsible one is shown by the experiment illustrated in Fig. 1c. Note that the activity appears normal despite the absence of all the central nuclei on both sides.

A second possible reverberating circuit is Purkinje cell (A) through Purkinje cell axon collateral (I) back to a second Purkinje cell. Thus, one Purkinje cell could fire another, then a third, etc., and back again. Although our experiments do not disprove the possibility of this circuit functioning in this manner, as can be seen below, there are other more tenable explanations.

*Spontaneous activity of cellular aggregates.* The most conspicuous cell in the cerebellar cortex is the Purkinje cell. The large soma,

mortality ratio was 5/14 in the Phenergan-treated animals, compared to 11/14 in those receiving 0.55 - 0.6 mg per kg of epinephrine without Phenergan. In the group to which 1.2 - 1.5 mg per kg of epinephrine was administered, 8 of 11 Phenergan-treated animals died.

Our results, as well as those of Stone and Loew, do not lend support to Halpern's hypothesis that Phenergan opposes the forma-

tion of pulmonary edema by virtue of the property of inhibiting cellular permeability.

*Summary.* The antihistaminic drug, Phenergan (3277 R.P.), in doses of 20 or 40 mg per kg, failed to protect rats or guinea pigs against the pulmonary edema induced by injection of ammonium chloride, and also offered no protection against epinephrine-induced pulmonary edema in guinea pigs.

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## Maintenance of Spontaneous Activity within the Cerebellum.\*† (17352)

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The hitherto unsuspected widespread projection of the cerebellum<sup>1</sup> to the cerebral cortex and the ease with which facilitation<sup>2</sup> and suppression<sup>3</sup> of cortically and reflexly induced movements can be produced by cerebellar excitation emphasizes the need to answer the interesting but intricate question, what is the cerebellar influence on the rest of the nervous system? One approach to an answer is the study of mechanisms underlying the maintenance of the fast electrical activity of the cerebellum. The best study yet made on the nature of this electrical activity was that by Dow<sup>4</sup> who not only described it in great detail, but also studied the influence of various drugs on it and related the activity to muscular tone.

In the present study we have confirmed many of Dow's observations and extended the problem into an analysis of intrinsic cerebellar mechanisms which maintain what is probably

the fastest electrical activity within the nervous system.

*Methods.* In mature cats, decerebrated, or placed under ether, nembutal, dial, chloralose, or various combinations of the above mentioned anesthetics, the calvarium was removed to expose all parts of the cerebellum except the anterior one-half of anterior lobe and paraflocculus and flocculo-nodular lobe. Bipolar and monopolar silver wire as well as saline-cotton wick pick-up electrodes were used to introduce the cerebellar activity into Grass Model 111 amplifiers. The amplified electrical activity was observed and photographed on a five-inch cathode ray tube.

*Results.* The first question was: Does this fast electrical activity result from the driving of the cerebellum by extrinsic centers or is it something intrinsic to the cerebellum? As shown in Fig. 1a, the activity results from intrinsic mechanisms within the cerebellum. The electrical activity in this preparation was normal despite the fact that 5 days previous all afferent and efferent fibers (verified histologically) had been cut. Previously, Dow<sup>4</sup> reported fast electrical activity following "acute incomplete section of the cerebellar peduncles in four experiments" and Spiegel<sup>5</sup> also observed it

Further support for this observation is given

\* This research was presented before the American Physiological Society, Sept. 1948.

† This research was aided financially by a grant from the Office of Naval Research.

<sup>1</sup> Henneman, E., Cooke, P., and Snider, R. S., *Am. J. Physiol.*, 1948, 155, 443.

<sup>2</sup> Snider, R. S., and Magoun, H. W., *Med. Proc.*, 1948, 7, 117.

<sup>3</sup> Snider, R. S., Magoun, H. W., and McCulloch, W. S., *Med. Proc.*, 1947, 6, 207.

<sup>4</sup> Dow, R. S., *J. Physiol.*, 1938, 94, 67.

<sup>5</sup> Spiegel, E. H., *Am. J. Physiol.*, 1937, 118, 569.

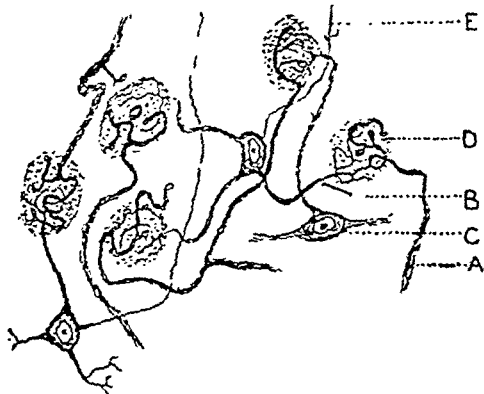


FIG. 3.

Outline drawing of microscopic anatomy of granule cell and granule cell glomerulus (modified from Cajal). A, Afferent fiber terminating as mossy fiber in protoplasmic glomerulus, D. C, Granule cell body. B, Dendrite of granule cell synapsing with afferent fiber in granule cell glomerulus. E, Granule cell axon.

cellular elements of the cerebellar cortex. We were not able to accomplish this technical procedure.

The Purkinje cell is not the only potential source of cellular elements for the maintenance of spontaneous electrical activity. A second possible source is the granule-basket cell combination in which the granule cell glomerulus deserves some comment. This non-nucleated protoplasmic structure located at the junction of the incoming mossy terminal with the dendritic terminals of the granule cells is approximately  $15\ \mu$  in diameter. Fig. 3 illustrates the salient properties of the various parts of the granule cell. Note that the incoming afferent fiber (A) synapses within the

glomerulus (D) on the way to the granule cell (C) dendrite (B). Note also that, except for a slightly granulated appearance, the protoplasm appears almost structureless.

To check the possibility of activity coming from the granule cell layer, we attempted to record directly from this layer with pick-up wire (26 gauge, insulated to tip). Although good activity could be recorded, and often better activity than that from the pial surface, which is approximately  $250\ \mu$  from the granular layer, the answer is still equivocal because possible activity of nearby Purkinje cells may have spread to the needle tip or Purkinje cell axons passing nearby may have been conducting activity through the area to the underlying cerebellar nuclei. From this, one must conclude that if the intrinsic spontaneous electrical activity of the cerebellar cortex results from spontaneous activity of cellular aggregates, as seems likely, then the basket-granule cell Fig. 2 D,F (with glomerulus) combination appears to be a possible responsible agent. One thing is certain, either it is this cellular complex or it is the Purkinje cell itself which is responsible.

**Summary.** Evidence is presented to show: (a) that the fast electrical activity of the cerebellum is intrinsically maintained within this structure; (b) that it is not due to reverberating circuits through the cerebellar nuclei and back to the cerebellar cortex; (c) that it is probably due to the spontaneous activity of Purkinje cells and/or the granule-basket cell combination.

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### Prevention of Secondary Infection due to *Pseudomonas aeruginosa* in Frostbitten Tissue. (17353)

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One of the major problems encountered in investigations on experimental frostbite is the prevention of infection of the frozen tissue. Without prophylactic treatment infection occurs in practically all cases of severe frostbite.

The following report gives the results of the application of penicillin and sulfamylon\*

\* The sulfamylon hydrochloride powder was supplied by the Medical Research Department of Winthrop-Stearns, Inc.



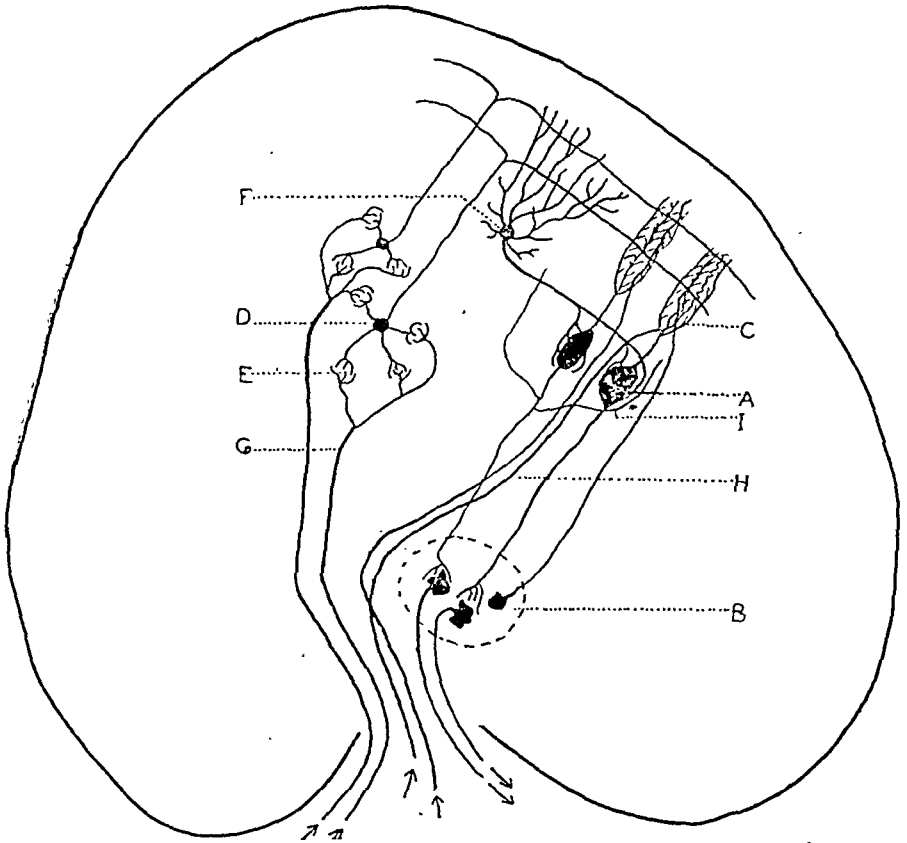


FIG. 2.

Diagrammatic drawing of microscopic anatomy of cerebellum. Modified from Ranson, 1935, *The Anatomy of the Nervous System*. A, Purkinje cell body. B, Cell body in central cerebellar nuclei. C, Purkinje cell dendrites. D, Granule cell body. E, Glomerulus in which afferent fiber mossy terminal synapses with granule cell dendrite. F, Basket cell body. G, Afferent fiber terminating as mossy terminal. H, Incoming fiber terminating as climbing fiber. I, Purkinje cell axon collateral.

the elaborate branching dendrites, and the axonal connections to the central nuclei make it one of the important cells, and one of the first to be considered as a possible source of spontaneous activity. This cell is of further interest because of the spatial representation of other cellular components on its cell body and processes. The basket cell axons synapse around the cell body, while the granule cell (Fig. 2, D) axons and climbing fibers (Fig. 2, H) synapse on the Purkinje cell dendrites. Possible synapse points on Purkinje cell axons are not known. As is well known, granule cell axons connect with Purkinje cells along the longitudinal axis of the folium and basket cell

axons connect with them along the transverse axis of the folium. In this manner, impulses coming from granule cells can be widely dispersed in the longitudinal and transverse planes of the cerebellar cortex and, from Purkinje cells, can be relayed in turn to the central cerebellar nuclei thence to centers within the brain stem. Thus, one cannot doubt the major role played by these cells in relaying the message through the cerebellar cortex. However, in order to study the role played by these cells in the maintenance of the fast electrical activity, according to the techniques we were using, it would be desirable to selectively destroy them, leaving intact the other

TABLE III.  
Comparison of the Effect of Local Treatment with  
Penicillin and with Sulfamylon.

	Number	Not infected	Infected
Penicillin treated	78	48	30
Sulfamylon treated	212	208	4
Total	290	256	34

$$\chi^2 = 70.2 \text{ and } P = < 0.0001.$$

imals were treated in exactly the same manner as the preceding group, except that instead of penicillin ointment an ointment with 3% sulfamylon was used. Of these animals 60 did not receive any other treatment while 120 were heparinized. Signs of local infection did not occur in these animals.

Another group of 32 rabbits was frostbitten in the same manner as in the preceding series. They were used for temperature measurements during freezing and thawing and received little care thereafter. They were routinely treated by local application of sulfamylon ointment, but the dressings were usually changed every second to fourth day. In this group 4 animals developed local infection at the site of injury in spite of the application of sulfamylon. Remarkably enough these infections were found after the dressings had not been checked for 3 days.

Even if we include the separate group of 32 animals with inadequate treatment, in which the only infections during sulfamylon treatment occurred, the results show undoubtedly the superiority of sulfamylon.

The statistical computation of these results shows that the difference is highly significant ( $\chi^2 = 70.2$  and  $P = < 0.0001$ ).

**Discussion.** Since the introduction of p-aminomethylbenzenesulfonamide (marfanil or sulfamylon) it has been shown to be a very effective drug in the prevention and cure of local infection. It is effective against a most diversified number of organisms in clinical as well as laboratory tests.<sup>1,3,5-10,13</sup> Its ef-

fectiveness against *Pseudomonas aeruginosa* (*B. pyocyaneus*) which caused the infections in our frostbite experiments is of special interest. Clinical infections with *Pseudomonas aeruginosa* respond well to treatment with p-aminomethylbenzenesulfonamide.<sup>2,5,6,9</sup> The same results were obtained in bacterial culture tests.<sup>6,8</sup> We agree with Howes<sup>6</sup> that this drug is distinctly superior to penicillin in its effectiveness against *Pseudomonas aeruginosa*. But since the effect is bacteriostatic,<sup>6,8</sup> it seems necessary to continue the treatment regularly until the possibility of infection has passed. The usefulness of p-aminomethylbenzenesulfonamide in the prevention and cure of infection in frostbite has already been reported in former papers.<sup>11,14</sup> It is also effective in the treatment of deep burns<sup>4</sup> where similar tissue conditions exist.

**Summary and conclusions.** 1. Of 78 animals with severe experimental frostbite 30 cases of infection with *Pseudomonas aeruginosa* (*B. pyocyaneus*) occurred, although the frozen area was treated locally with penicillin ointment.

2. Of 212 animals with the same degree of cold injury and treated with 3% sulfamylon ointment, only 4 animals developed local infection with *Pseudomonas aeruginosa*. In 180 of these animals the dressings were changed daily and none became infected. The dressings of 32 animals were changed every 2 to 4 days; in this group 4 infections occurred.

3. Sulfamylon is superior to penicillin in its effect on *Pseudomonas aeruginosa* infections.

4. It is possible to control the infection due

<sup>6</sup> Howes, E. L., *Surg., Gynec., and Obst.*, 1946, **83**, 1.

<sup>7</sup> Klarer, J., *Klin. Wschr.*, 1941, **2**, 1250.

<sup>8</sup> Lawrence, C. A., *J. Bact.*, 1945, **49**, 149.

<sup>9</sup> McLaurin, J. W., *Laryngoscope*, 1948, **58**, 1201.

<sup>10</sup> Mitchell, G. A. G., Rees, W. S., and Robinson, C. N., *Lancet*, 1944, **1**, 627.

<sup>11</sup> Moser, H., *Dtsch. Med. Wschr.*, 1942, **1**, 549. Abstract in: *Bull. War Med.*, 1942-1943, **3**, 255.

<sup>12</sup> Pichotka, J., and Lewis, R. B., in press.

<sup>13</sup> Schreus, H. Th., *Klin. Wschr.*, 1942, **2**, 671.

<sup>14</sup> Wojta, H., *Der Chirurg*, 1943, **15**, 85. Abstract in: *Bull. War Med.*, 1943-1944, **4**, 12.

<sup>1</sup> Beyer, W., *Zentr. Chir.*, 1941, **68**, 1730.

<sup>2</sup> Clark, W. B., *Arch. Oph.*, 1947, **38**, 682.

<sup>3</sup> Domagk, G., *Dtsch. Med. Wschr.*, 1943, **69**, 379.

<sup>4</sup> Editorial, *Lancet*, 1944, **1**, 635.

<sup>5</sup> Fox, S. L., *Ann. Otol., Rhin., and Laryng.*, 1947, **56**, 946.

TABLE I.  
Incidence of Infection in Frostbitten Legs Treated  
Locally with Penicillin.

	Number	Not infected	Infected
Heparin treated	42	32	10
Non-heparin treated	36	16	20
Total	78	48	30

$\chi^2 = 6.82$  and  $P = 0.008$ .

TABLE II.  
Incidence of Gangrene in Infected and Non-infected Animals Treated with Penicillin.

	Number	Completely saved	Partially saved	Completely lost	Died
Non-infected	48	0	11	18 (2)*	19
Infected	30	0	4	22	4

\* Number in parentheses indicates animals which died after the fate of the leg was decided.  
 $\chi^2 = 2.00$  and  $P = 0.15$ .

(p-aminomethylbenzenesulfonamide) to the injured area after severe experimental local cold injury.

The experiments were performed on 290 male white albino rabbits weighing 2,000 to 3,000 g. The injury was produced by immersing one depilated hind leg for 30 minutes in an alcohol bath cooled with dry ice to temperatures ranging from  $-10^{\circ}\text{C}$  to  $-25^{\circ}\text{C}$ . The exact experimental procedure is described in another paper.<sup>12</sup> After the experiment the frozen area was covered with wool fat and a sterile dressing applied. During the first week the dressings were changed every day, sometimes every other day. Infections usually became evident between the fourth and sixth day after injury. The infected frozen area appeared bluish green, swollen, and slightly translucent. Small hemorrhages in the parts bordering the healthy tissue were quite often present. Pus oozed from the injured area, especially from the region of the demarcation line. From the bluish discoloration of the bandages and from the characteristic odor it was assumed that the infection was caused by *Bacillus pyocyaneus*. The bacteriological examination of 2 cases revealed pure cultures of *Pseudomonas aeruginosa* (*B. pyocyaneus*). The clinical appearance was characteristic enough that no further bacteriological investigation was considered necessary.

Of 290 animals 162 received intravenous

heparin therapy and 128 did not. The occurrence of gangrene was practically the same in the two series. Of the entire group 48 saved the frostbitten leg completely, 42 lost part of the injured limb, 105 lost the leg completely, and 95 died, 14 of them after the final fate of the leg was decided.

*Penicillin.* Seventy-eight animals, heparinized and non-heparinized, received local treatment with penicillin ointment (1,000 units per

cc). In this group 30 animals showed signs of infection in the frostbitten area and 48 did not.

The degree of injury was the same in both groups of animals. The difference in the incidence of infection in the heparinized and non-heparinized animals is significant ( $\chi^2 = 6.82$  and  $P = 0.008$ ). We do not have an explanation for this finding. We do not believe the evidence sufficient to support the conclusion that heparin is effective in preventing local infection of frostbitten tissue. The incidence of gangrene of the frostbitten legs in the infected and non-infected animals shows no essential differences (Table II). The higher death rate in the non-infected group is mainly due to the fact that most of the deaths occurred before the fourth or fifth day when the infections usually became evident. The relation of partially lost to completely lost legs is slightly less favorable for the infected animals ( $\chi^2 = 2.00$  and  $P = 0.15$ ). But even if this difference were significant it would not necessarily mean that it was due to the infection. According to our observations the occurrence of infection of frostbitten tissue in rabbits is related to the diarrhea that develops in some animals after cold injury. There seems to be a higher probability for the appearance of diarrhea with increasing severity of cold injury.

*Sulfamylon.* One hundred and eighty ani-

TABLE I.  
Results of Heparin Treatment in Very Severe Frostbite.

No. of animals	Treatment	No necrosis	Partial necrosis	Complete necrosis	Died	Survived
12	2 cc heparin every 4 hr	0	3	2	7	5
30	3 cc heparin every 6 hr	0	4	23 (1)*	4	26
39	3 cc heparin every 12 hr	0	1	12	17	22
Total—81		0	8	46 (1)*	28	53
Controls—42		0	7	28 (2)*	9	33

\* Number in parentheses indicates animals which died after fate of the leg was decided and which therefore appear twice in the table.

123 animals frozen in an alcohol bath at  $-25^{\circ}\text{C}$  with rubber boot loosely attached to leg.

81 animals were treated with heparin for 6 days.

42 animals were controls.

The distribution of "partial necrosis" and "complete necrosis" is essentially the same in both groups. ( $\chi^2 = 1.2$  and  $P = 0.27$ ).

The death rate is not significantly different. ( $\chi^2 = 1.7$  and  $P = 0.18$ ).

covered with sterile dressings and the animals put into individual cages. Generally the dressings were changed every day. The animals which showed severe swelling had the first dressing changed after 12 hours to prevent obstruction of the circulation by a tight bandage. At the end of the first day the swelling was at its peak, and thereafter the danger from constrictive dressings was negligible. We originally used a thin layer of wool fat to prevent adherence of the bandages due to the oozing of plasma. After some infections occurred, we added penicillin to the wool fat, but without success. Subsequently sulfamylon was used which eliminated further infection in the course of the investigation. The prevention of infection following experimental frostbite is the subject of a separate paper.<sup>9</sup>

In the second part of the investigation the conditions of exposure were changed since we felt it necessary to use a less severe cold injury to evaluate heparin therapy.

The experiments were performed from February 1948 to March 1949 on 315 animals. Of these, 153 animals received heparin\* for 6 days, 80 served as controls, and 82 were used for the determination of a degree of in-

jury suitable for the evaluation of the treatment.

The statistical method used is a test of significance using  $\chi^2$  (chi-square) in two-way contingency tables.

*Repetition of the Experiments of Lange and Collaborators.* Lange and his co-workers used different temperatures and exposure times in their animal experiments.<sup>2,5</sup> The largest group with homogeneous conditions consisted of 41 animals of which one hind leg was exposed to a bath of  $-30^{\circ}\text{C}$  ( $-22^{\circ}\text{F}$ ) for 30 minutes.<sup>7</sup> Twenty-one of these animals received 3 cc of heparin intravenously every 12 hours for 6 days. In a personal communication Lange advised us to give 3 cc of heparin every 6 hours.

The corresponding group in our experiments consisted of 123 animals. One hind leg was depilated, loosely covered with a rubber boot, and immersed to the knee joint in a bath of  $-25^{\circ}\text{C}$  ( $-13^{\circ}\text{F}$ ) for 30 minutes and then allowed to thaw at room temperature. Eighty-one of these animals received heparin and 42 served as controls. The heparin was administered by 3 different regimes as shown in Table I. The initial injection of heparin was made from 1 to 4 hours after freezing in 51 animals, 4 to 8 hours in 14, and 8 to 12 hours in 16.

Blood coagulation times were determined

<sup>9</sup> Pichotka, J., and Lewis, R. B., to be published.

\* The heparin used was liquesmin (Roche-Organon, Inc.) and heparin (Abbot).

to *Pseudomonas aeruginosa* in experimental frostbite by daily local application of sulfamylon.

We wish to give credit to Mr. Allyn Kimball of

the Department of Biometrics, School of Aviation Medicine, Randolph Field, Texas, for making the statistical analyses of the experimental data.

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## Use of Heparin in Treatment of Experimental Frostbite. (17354)

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During World War II casualties from local cold injury were numerous. In some units they exceeded the number of casualties from every other cause. This was especially true of Air Force personnel on high altitude bombing missions. It was therefore quite natural that much interest was focused on the investigation of the mechanism and therapeusis of cold injury.

Among the publications on the treatment of local frostbite, the reports of Lange and his collaborators were the most remarkable.<sup>1-5</sup> These authors described the results of heparin in the prevention of gangrene in animals and human volunteers after local cold injury. The control cases in their experiments always developed extensive or complete necrosis of the frostbitten area whereas the heparin treated cases almost always escaped gangrene. The details of the experimental procedures and of heparin administration as used in their investigations will be discussed later.

Publications subsequent to those of Lange *et al.* did not confirm their results.<sup>6,7,8.</sup> Be-

cause of the importance of the subject to the Air Force we deemed it advisable to repeat these experiments.

**Material and Method.** With few exceptions in the early series we used white male albino rabbits of more than 2,000 g body weight. Female animals were excluded since in one series they seemed to be more susceptible than males to internal hemorrhages after heparin administration. The animals were kept in the open air throughout the year, but at least one week before the experiment they were brought into the air conditioned animal house and kept at 25°C (77°F). The food consisted of Purina rabbit pellets and occasionally carrots; water was unlimited. In the course of the investigation during which more than 300 animals passed through our animal room only 2 animals died spontaneously.

During the first part of the experiment we followed the procedure of Lange *et al.*<sup>2,5</sup> as closely as possible, except that the animals were not anesthetized during freezing. The right hind leg was clipped and depilated with Zip Depilatory Cream from the ankle upwards and loosely covered with a rubber condom boot to about 2 cm to 3 cm above the knee joint. The prepared hind leg was immersed to the knee joint in a bath at -25°C (-13°F) for 30 minutes. The freezing bath consisted of 95% alcohol cooled with dry ice. After some experience it was possible to keep the bath within 0.5°C of the desired temperature. Immediately after freezing, the legs were

<sup>1</sup> Friedman, N. B., Lange, K., and Weiner, D., *Am. J. M. Sc.*, 1947, **213**, 61.

<sup>2</sup> Lange, K., and Boyd, L. J., *Surg., Gynec., and Obst.*, 1945, **80**, 346.

<sup>3</sup> Lange, K., and Loewe, L., *Surg., Gynec., and Obst.*, 1946, **82**, 256.

<sup>4</sup> Lange, K., Boyd, L. J., and Loewe, L., *Science*, 1945, **102**, 151.

<sup>5</sup> Lange, K., Weiner, D., and Boyd, L. J., *New England J. Med.*, 1947, **237**, 383.

<sup>6</sup> Fuhrman, F. A., and Crismon, J. M., *J. Clin. Invest.*, 1948, **27**, 364.

<sup>7</sup> Quintanilla, R., Krusen, F. H., and Essex, H. E., *Am. J. Physiol.*, 1947, **149**, 149.

<sup>8</sup> Schumacker, H. B., Jr., White, B. H., Wrenn, E. L., Cordell, A. R., and Sanford, T. F., *Surgery*, 1947, **22**, 900.

TABLE II.  
Results from Exposure for 30 Minutes at Different Temperatures without Treatment.

Bath temp.	Avg deep muscle temp.	No necrosis	Partial necrosis	Complete necrosis	Died	No. animals
-10°C	-3.3°C	25	1	0	0	26
-15°C	-7.4°C	4	16 (1)	12 (1)	8	38

TABLE III.  
Results of Heparin Treatment in Less Severe Frostbite.

No. animals	No necrosis	Partial necrosis	Complete necrosis	Died	Survived
Treated—72	3 (2)	21 (6)	22 (12)	46	26
Controls—38	4	16 (1)	12 (1)	8	30

110 animals exposed at -15°C for 30 minutes with rubber boot adherent to the leg.

72 animals received 3 cc of heparin every 6 hours for 6 days.

38 animals were controls.

The incidence of necrosis (partial and complete) is the same in both groups. ( $\chi^2 = 0.26$ ,  $P = 0.62$ ).

The distribution of the results of "no necrosis," "partial necrosis," and "complete necrosis," is essentially the same. ( $\chi^2 = 0.46$  and  $P = 0.95$ ).

The increase in the death rate of the treated animals is highly significant. ( $\chi^2 = 16.6$  and  $P = <0.0001$ ).

frostbite necrosis.

One hundred and ten animals were subjected to freezing at -15°C for 30 minutes; 72 of these were treated with 3 cc of heparin every 6 hours. The results are given in Table III.

The statistical evaluation of the results of the injury as determined by the chi-square test, including those cases in which the fate of the leg was determined at the time of death, shows that there is no significant difference between treated and untreated animals insofar as the incidence of necrosis is concerned ( $\chi^2 = 0.256$  and  $P = 0.62$ ). There is also no difference in the degree of final injury. If the results are computed with regard to the distribution of cases with "no necrosis," "partial necrosis," and "complete necrosis,"  $\chi^2 = 0.46$  and  $P = 0.95$ . On the other hand the death rate of the treated animals is significantly increased. ( $\chi^2 = 16.6$  and  $P = <0.0001$ .)

*Fatalities During Heparin Treatment.* As already mentioned, the fatalities in the heparin-treated groups were much more numerous than in the corresponding controls. Of 153 heparin-treated animals 74 were lost during the 6 days of treatment whereas of 80 controls only 17 animals died. The difference in the death rate between these two groups

is highly significant ( $\chi^2 = 15.2$  and  $P = 0.0001$ ). The fatalities in the heparin-treated series can be divided into two main groups. In one group, the animals exhibited a shock-like condition after the first or second injection of heparin without hemorrhages. The second group consisted of fatal hemorrhages which occurred in the course of the treatment.

It was soon apparent that fatalities without hemorrhages after the first injection of heparin were directly related to the time which elapsed between injury and heparin injection. This is shown for 72 cases under homogeneous experimental conditions and with intervals between injury and first injection of from 0-12 hours (Table IV). The increase in death rate occurring with increasing intervals between

TABLE IV.  
Death Rate of Animals in Relation to the Time Interval Between Cold Injury and the First Injection of Heparin.

Interval between injury and first injection	Survived	Died	Total
0-2 hr	22	0	22
2-4 "	16	1	17
4-8 "	10	7	17
8-12 "	8	8	16

The statistical evaluation shows that the increased death rate with increasing interval between injury and first heparin injection is significant. ( $\chi^2 = 20.6$  and  $P = 0.00015$ ).

in 28 experiments following the injection of 2 cc of heparin intravenously. The capillary method was used; blood samples were obtained from the ear veins by means of nicking with a sharp needle. Intervals between determinations were 10 minutes in one series and 30 minutes in another.

The results of this experiment do not show any benefit of heparin treatment. In both groups all animals developed tissue necrosis. The statistical evaluation of the observed frequencies of *partial and total necrosis in treated and untreated animals* shows a value for  $\chi^2$  of 1.2 and for P of 0.27. Hence the results in the two groups are not significantly different. The death rate for the heparin treated animals is slightly greater than for the controls but the difference is not significant ( $\chi^2 = 1.7$  and  $P = 0.18$ ). Table I.

*Use of Heparin in Less Severe Frostbite.* Since our results were not in agreement with those of Lange *et al.* we sought reasons for the discrepancy. The most probable explanation for the differences seemed to be that we did not produce the same degree of injury in our experiment that Lange had in his. Therefore, we first had to standardize our method of producing cold injury. The use of the rubber boot as described by Lange is a possible source of error. Fixation by longitudinal strips allows a considerable amount of air to remain between the leg and rubber boot, which might vary considerably in amount with different sizes, shapes, or positions of the legs. The heat conductivity is considerably altered by the layer of air around the leg and the degree of this change depends on the quantity of air in the boot.

For practical reasons experiments on frostbite with short exposure times must be performed in a fluid with a low freezing point, such as alcohol. Alcohol has an injurious effect on the skin as we could observe in our experiments. Therefore, it is not possible to escape the use of a protective covering which would have been the simplest way to avoid difficulties from altered conductivity.

We overcame this difficulty by attaching the rubber boot as closely to the leg as possible. After the boot was pulled over the foot and

fixed loosely with circular adhesive strips at the ankle, the air was pressed out of the boot. The entire depilated limb was then covered with a thin layer of wool fat and the rubber boot pulled over the leg thus making it adherent to the skin. Finally the boot was fixed above the knee by a broad circular strip of tape and at the ankle with a narrow adhesive band. Folds in the rubber boot which sometimes could not be avoided were moved to the posterior aspect of the leg.

The legs of animals prepared in this way were immersed for 30 minutes in a bath of from 0°C to -25°C (+32°F to -13°F) with intervals of 5°C. The actual temperatures in different depths of the exposed leg were recorded simultaneously throughout the exposure and period of thawing.<sup>10</sup>

Judging from the clinical results and from the recorded temperatures inside the leg, the experiments with the rubber boot closely adherent were reproducible to a high degree.

For an exposure time of 30 minutes the temperature range of the freezing bath between -10°C and -15°C (+14°F to +5°F) was of greatest interest (Table II). At -10°C (+14°F) 25 of 26 animals did not develop necrosis and one showed superficial gangrene of the frozen limb; at -15°C (+5°F) 4 animals of 38 showed no necrosis, 16 partial necrosis, and 12 complete loss of the frozen leg. Six died before the fate of the frozen leg was decided. This distribution indicates that the injury of the group exposed at -15°C (+5°F) was just past the borderline from which spontaneous recovery was not possible for the overwhelming majority of our animals. We therefore assumed that any beneficial effect of heparin in the prevention of gangrene after frostbite would become evident under these conditions.

The actual temperatures in the deep muscle recorded in this group during exposure are much higher than in those immersed for 30 minutes at -25°C. On the average they barely passed the range of -6° to -7°C considered by Lake<sup>11</sup> to be decisive for the occurrence of

<sup>10</sup> Pichotka, J., and Lewis, R. B., to be published.

<sup>11</sup> Lake, N. C., *Lancet*, 1917, 2, 557.

TABLE V.  
Incidence of Necrosis in Relation to Degree of Injury.\*

°C for 30 min.	Temp. in deep tissue (No. of measurements in parentheses) °C	No necrosis	Partial necrosis	Complete necrosis	Total
0	+6.8 (2)	12	0	0	12
-5	+2.2 (2)	12	0	0	12
-10	-0.3 (7)	25	1	0	26
-12†	-3.3 (6)	27	5	0	32
-15	-7.4 (14)	4	16	12	32

\* Animals which died before the fate of the tissue was decided are not considered.

† These animals were exposed to a bath temperature of  $-15^{\circ}\text{C}$  with different conditions of conductivity (rubber boot not adherent). By measurement of the temperatures in the leg and by interpolation we determined this temperature to correspond to  $-12^{\circ}\text{C}$  with the higher conductivity (rubber boot adherent).

striking. While at  $-12^{\circ}\text{C}$  only 5 of 32 showed tissue necrosis, at  $-15^{\circ}\text{C}$  28 of 32 suffered gangrene. The distribution of the degree of injury in the group exposed to  $-15^{\circ}\text{C}$ , i.e., 12 cases of total gangrene, 16 cases of partial gangrene, and 4 cases without necrosis, should furnish an ideal basis for the evaluation of treatment of acute cold injury. The beneficial effect would become apparent in a shift to the groups of lesser injury.

As shown in Table III, the statistical computation of treated and untreated animals with this degree of injury does not show such a shift. There is significantly no difference in the distribution of the degree of injury between the two groups ( $P = 0.95$ ).

Several other investigators who attempted to repeat Lange's experiments came to the same conclusions as we did. Quintanilla *et al.*<sup>7</sup> were not able to demonstrate any beneficial effect of heparin after various degrees of frostbite. Fuhrman and Crismon<sup>6</sup> in their experiments also did not obtain favorable results from heparin treatment.

It is difficult to explain the failure of several investigators to reproduce Lange's results. The only major point of Lange's experimental procedure which we did not follow was in the use of anesthesia. But Fuhrman and Crismon,<sup>6</sup> Schumacker *et al.*,<sup>8</sup> Quintanilla *et al.*,<sup>7</sup> anesthetized the animals before freezing and did not obtain better results.

The assumption that Lange *et al.* actually produced less severe injuries in their experiments than we did in reproducing them does not abolish this discrepancy. In the second phase of our experiments heparin proved to

be ineffective even in the least degree of cold injury that is followed by gangrene. There remains the possibility that some of the factors not sufficiently controlled, such as species of animals used, food, acclimatization, temperatures at which the experiments were performed or at which the animals were kept during the time of observation, might prove to be of major importance.

The incidence of fatalities due to heparin treatment was of secondary interest in our investigations. However, it must be considered in the evaluation of heparin as a therapeutic agent. Furthermore, the death rate determines very much the value of the statistical analysis of the therapeutic results.

The statistical computation applied to our data is not correct in a strict sense. The results were tabulated under the headings of "no gangrene", "partial gangrene", "total gangrene", and "death". These groups are not comparable from a statistical point of view. We can reasonably assume that "no gangrene", "partial gangrene", and "total gangrene" constitute a sequence of increasing severity. Death of the animals either with or without treatment is not comparable to the other groups. Death occurred when, according to the clinical appearance, the injured leg was completely lost, or partially lost, or even completely saved. In 2 large groups we observed that the fatal outcome was due to the delayed first injection of heparin or to seasonal changes in sensitivity to heparin. We do not see how it is possible to determine whether the probability of dying or surviving under these conditions is or is not the same for the three



injury and first injection is significant ( $\chi^2 = 20.6$  and  $P = 0.00015$ ).

The deaths from internal hemorrhages were far more numerous than those of the first type. Fifty-three of 153 heparin treated animals died from fatal hemorrhages into the peritoneal cavity, the retroperitoneal space, or into the lungs. The amount of heparin given before death occurred from hemorrhages differed greatly. Some animals developed hemorrhages after the first injection of 30 mg of heparin, others after more than 20 injections. On the average the animals which died from acute hemorrhages received 300 mg of heparin in a period of 60 hours by a regime of either 2 cc every 4 hours or 3 cc every 6 hours.

There was a distinct seasonal difference in the occurrence of hemorrhages during heparin treatment. Fatal hemorrhages occurred more often and with much smaller doses during the winter months. The reason for this observed seasonal difference is not apparent at this time.

*Discussion.* A comparison of our results with those of Lange *et al.* is possible only if the cold injury is determined by the physical conditions under which it occurs. We have shown in another investigation that under conditions which produce gangrene as a result of acute local cold injury, the immersed hind leg of a rabbit behaves practically like a dead body.<sup>10</sup> The time course of the temperatures in the exposed limb and the clinical results are very much the same in all animals investigated under the same standardized conditions. We should therefore be able to reproduce a comparable degree of cold injury by applying the same experimental conditions.

The cold injury in Lange's experiment was produced by two different procedures. One group of animals was exposed for 45 to 90 minutes to an alcohol dry ice bath which varied in temperature between  $-12^\circ\text{C}$  and  $-20^\circ\text{C}$ ;<sup>2</sup> the other group was frozen for 30 minutes at  $-30^\circ\text{C}$ .<sup>5</sup>

The data concerning the first group are not exact enough to permit reproduction. The extreme possibilities for these conditions include almost 100% probability for complete preservation of the immersed legs as well as 100%

probability for complete loss. The second regime, *i.e.*, exposure for 30 minutes at  $-30^\circ\text{C}$ , invariably produces very severe frostbite. But since Lange in a personal communication informed us that the experiments were conducted between  $-20^\circ\text{C}$  and  $-30^\circ\text{C}$ , and since in a control series at  $-25^\circ\text{C}$  very severe frostbite always occurred, we used this latter temperature for the first part of our experiments. During this part of the experimental work the rubber boot was loosely attached by longitudinal strips, as described in the papers of Lange *et al.*<sup>2</sup> We failed completely to find any beneficial influence of heparin. Treated and untreated animals showed the same results. The number of animals was large enough to exclude the possibility that the results were due to random variation. (Table I.) We cannot give a reasonable explanation for the differences between Lange's and our results.

The maintenance of the blood coagulation time between 30 and 60 minutes is not possible when heparin is given intravenously at 12 or 6 hour intervals as described in Lange's publications. In 28 experiments we administered 2 cc of heparin intravenously to rabbits weighing 2,000-3,000 g. Blood clotting times were prolonged to 8 or more hours immediately, but within a few minutes they dropped sharply to reach the normal range within 4 hours or less. More exact investigations on the action of heparin will be published in another paper.

A more accurate evaluation of heparin in the prevention of frostbite gangrene was possible when we were able to perform the experiments with a degree of injury which did not preclude recovery. Systematic investigations showed that a feasible degree of injury could be obtained by an exposure to an alcohol bath of  $-15^\circ\text{C}$  for 30 minutes with the protective rubber boot closely adherent to the depilated leg.

The relation of occurrence of frostbite to degree of injury is shown in Table V. The progression of the severity of injury from a bath temperature of  $-12^\circ\text{C}$  to a bath temperature of  $-15^\circ\text{C}$  with deep tissue temperatures of  $-3.3^\circ\text{C}$  and  $-7.4^\circ\text{C}$ , respectively, is rather

permeable membrane. The *in vivo* studies were made by injecting a dye into the synovial cavity of rabbits and using the speed of appearance of dye in the urine and the rate of excretion into the urine as an index of permeability of the synovial membrane. These studies have been extended to human subjects under the influence of various drugs and diseases. In this paper we are presenting the data obtained *in vitro*. Those obtained *in vivo* correspond in nearly every detail to these and will be presented in a separate paper.

**Materials and methods.** The hyaluronidase\* was prepared from bull testes and assayed 7000 to 9400 turbidity reducing units per mg of nitrogen. Membranes used in this study were the lens capsule, urinary bladder membrane processed according to the method of Barnes,<sup>6</sup> and freshly prepared urinary bladder of the rabbit. We also studied the effects of hyaluronidase on water imbibition by skeletal muscle, penetration of drugs into excised frog hearts, and filtration through isolated frog skin, because membrane permeability may be involved in these phenomena.

**Crystalline lens membrane.** Freshly excised, intact lens of frogs and rabbits were used. Series of test tubes were set up, four in each series, with one pair containing distilled water and the other pair physiological salt solution. One lens was placed in each solution, the tube agitated, and an aliquot of the supernatant removed. To one tube of each pair hyaluronidase was added to make a 0.05% solution, the tubes again agitated, and samples removed. The tubes were maintained constantly at 38°C. The degree of turbidity of each solution was read every five minutes, using the B scale of the Fischer Electrophotometer. A water clear solution gave a light transmission of 100%; an in-

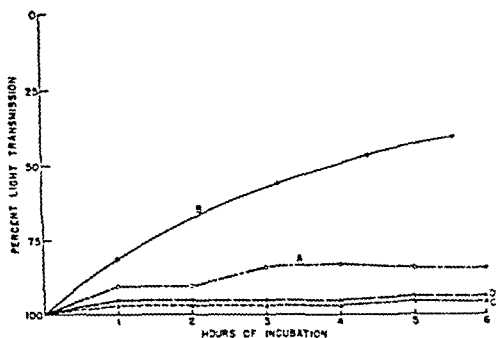


FIG. 1.

Turbidity of incubating medium containing whole rabbit lens (38°C). Fischer electrophotometer used for readings).

Curve A—Distilled water.

Curve B—0.05% hyaluronidase (assaying 9400 T.R.U. per mg N) in distilled water.

Curve C—Physiological salt sol.

Curve D—0.05% hyaluronidase (assaying 9400 T.R.U. per mg N) in physiological salt sol.

crease in turbidity decreased the amount of light transmitted (Fig. 1).

**Urinary bladder membrane.** The urinary bladder was removed surgically from rabbits by excision at the neck. Each membrane was prepared by washing and storing overnight at 36°C in 0.85% NaCl. The following morning a membrane was stretched and mounted over the open end of a thistle tube, the adventitia being toward the inside of the tube. After drying for 30 minutes in the hot air oven at 100°C, the membrane was waterproofed at the edge with paraffin and stored in the refrigerator until used. The preparation served as an osmometer with a semipermeable membrane separating M/1 sucrose on the inside from distilled water against the mucosal surface on the outside. One membrane could be used several times if after each experiment it were reprocessed by repeating the above procedure. When reprocessing was repeated on 5 separate membranes for 3 successive days the curves of the rate of osmosis obtained on the first day could be superimposed on those obtained on the third day, thus ruling out the possibility that reprocessing altered permeability. A total of 58 permeability experiments were done on 30 processed membranes.

Urinary bladder membranes maintained in the fresh state were also studied. After killing the rabbits by a sharp blow across the

\* Hench, P. S., Kendall, E. C., Slocumb, C. H., and Polley, H. F., *Proc. Staff Meet., Mayo Clin.*, 1949, **24**, 181.

<sup>5</sup> Hench, P. S., Slocumb, C. H., Barnes, A. R., Smith, H. L., Polley, H. F., and Kendall, E. C., *Proc. Staff Meet., Mayo Clin.*, 1949, **24**, 277.

<sup>6</sup> Hydase, Wyeth.

<sup>6</sup> Barnes, T. C., *Textbook of General Physiology*, 1937, Blakiston Co., Philadelphia, Pa.

groups of final injury. In other words, our material underwent a change and we do not know whether this change was selective or not.

Furthermore, we cannot give a numerical representation for the groups "no gangrene", "partial gangrene", "total gangrene". Our experimental results do not enable us to assume that the extent of necrosis is a continuous function of increasing severity of injury since all types of tissue do not show the same sensitivity to cold.

The statistical data given in the paper serve only the purpose of showing that the constellation of figures on which our conclusions are based are not due to random variation.

**Summary.** A total of 153 rabbits had their legs exposed to temperatures of  $-25^{\circ}\text{C}$  or  $-15^{\circ}\text{C}$  for 30 minutes and subsequently treated with heparin administered intravenously for a period of 6 days. Twelve animals received 20 mg of heparin every 4 hours, 39 received 30 mg every 12 hours, and 102 received 30 mg every 6 hours.

Eighty animals were frostbitten at the same temperatures and for the same exposure times and used as untreated controls.

A statistical analysis of the results showed that tissue necrosis from frostbite was not

significantly less in the heparin-treated animals.

Of 153 animals that were treated with heparin 74 died whereas 17 of 80 control animals succumbed during the experiment. The fatalities in the heparin-treated series were due to fatal internal hemorrhages in 53 cases; in 17 instances to a shock-like condition especially observed when the initial injection of heparin was delayed 4 or more hours after injury, and 4 animals died from unknown causes.

**Conclusions.** 1. The excellent results reported by Lange and his co-workers on the use of heparin in frostbite were not reproducible. 2. The results of heparin therapy by the regime recommended by Lange were not more satisfactory in our experiments than those in untreated control animals.

3. The death rate was significantly increased in animals that received heparin in doses proposed by Lange *et al.*

We wish to give credit to Mr. Allyn Kimball of the Department of Biometrics, School of Aviation Medicine, Randolph Field, Texas, for making the statistical analyses of the experimental data.

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### Alteration in Permeability of Some Membranes by Hyaluronidase and Inhibition of this Effect by Steroids. (17355)

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It is well established that hyaluronidase increases the "permeability" of the ground substance and can thereby markedly facilitate the spread of India ink, dyes, bacteria, drugs, etc. through the cutaneous tissues.<sup>1,2</sup> Hyaluronidase may also facilitate the penetration of some drugs into the cornea by attacking the monosulfuric ester of hyaluronic acid of this tissue.<sup>3</sup> It was of interest to investigate

the effects of hyaluronidase on the permeability of membranes both *in vitro* and *in vivo* since this has not been done heretofore. Such a study is of timely interest in light of the possible relationship of hyaluronidase as a causative factor in arthritis and the alleviation of arthritis by steroids such as Compound E.<sup>4,5</sup>

Most of the *in vitro* tests were made using the urinary bladder of the rabbit as a semi-

<sup>1</sup> Duran-Reynals, F., *Bact. Rev.*, 1942, 6, 197.

<sup>2</sup> Meyer, K., *Physiol. Rev.*, 1947, 27, 335.

<sup>3</sup> Seifter, J., *Ann. N. Y. Acad. Sci.*, in press.

TABLE I.  
Effect of Hyaluronidase and Steroids on the Permeability of Processed Membranes.

	No. of exp.	Avg rise at 360 min. (mm)	Range (mm)	Median (mm)
Distilled water	24	101	67-189	105
Hyaluronidase	10	245	130-360	240
Adrenal cortical extract*	3	91	62-124	87
Adrenal cortical extract + hyaluronidase	5	125	100-146	125
Cholesterol*	2	122.5	120-125	
Cholesterol + hyaluronidase	1	133		
Estrone*	2	110.5	110-111	
Estrone + hyaluronidase	2	50	29-71	
Testosterone*	1	93		
Testosterone + hyaluronidase	1	176		

\* These membranes had much greater permeability in the control period when only distilled water was used.

only in distilled water, since the released material might be soluble in physiological salt solution. Protein determinations of the supernatants were made by the sulfosalicylic acid method, and some of the data are presented in Fig. 2. Each curve was obtained by averaging the milligrams percent of protein for the supernatants of 6 to 8 lenses. The data for distilled water and distilled water-hyaluronidase were taken only from lenses showing blistering or rupture in one hour in order to make a comparison with physiological salt solution-hyaluronidase which for some as yet unexplainable reason became inactivated in one hour. Leakage of protein through the membrane immersed in distilled water or physiological salt solution did not occur to a significant degree during the period when the lens was intact (curves A and B). Hyaluronidase caused leakage of protein through the membrane during this period (curves C and D). Individual capsules differed markedly in their resistance to injury by the hypotonicity of distilled water and to alteration of permeability by hyaluronidase.

**Urinary bladder membrane.** The results on the processed membranes are listed in Table I and typified in Fig. 3. The rate of osmosis through a semi-permeable bladder membrane is shown by Curve A. The distilled water at the end of the experiment was sugar free by the anthrone method. Two  $\gamma$  of hyaluronidase per ml markedly increased the rate (Curve B). At the end of this experiment the concentration of sugar outside the membrane was 0.30 mg per ml as compared with 0.34 mg per ml

inside; therefore the semi-permeable nature was abolished. The permeability effect was reversible since removing the hyaluronidase

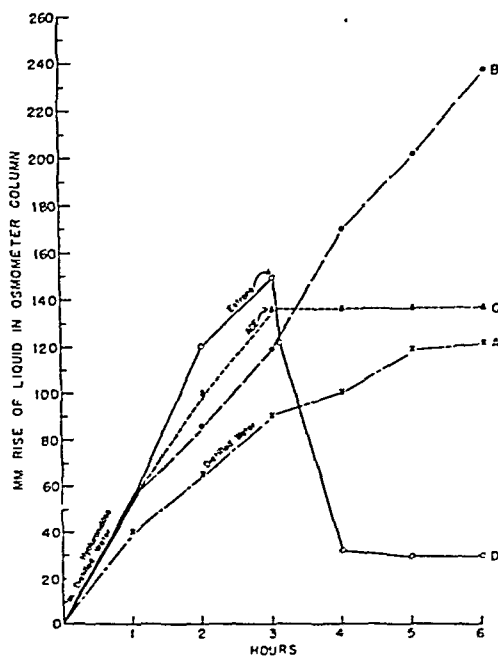


FIG. 3.

Rate of osmosis. (Urinary bladder membrane semi-permeable to M/1 sucrose).

Curve A—Distilled water outside osmometer.

Curve B—Hyaluronidase 1:100 (assaying 7000 T.R.U. per mg N) in distilled water (2  $\gamma$  per ml).

Curve C—Same concentration of hyaluronidase in distilled water as in curve B, but aqueous adrenal cortical extract, 10  $\gamma$  per ml, added at the arrow.

Curve D—Same concentration of hyaluronidase in distilled water as in curve B, but aqueous estrone, U.S.P., 10  $\gamma$  per ml, added at the arrow.

cervical vertebrae, the urinary bladder was immediately removed, washed in Tyrode's solution at  $37 \pm 1^\circ\text{C}$ , and by means of a purse string suture of surgical silk secured as quickly as possible to the open end of a thistle tube. The adventitia was inside and the mucosa outside. Tyrode's solution containing M/1 sucrose was placed within the osmometer which was immersed in a beaker containing Tyrode's solution. Oxygen was bubbled into the solution on each side of the membrane. A freshly excised bladder was used for each of 21 experiments performed at  $37^\circ\text{C}$  in a hot air incubator.

The following were tested for their effect on permeability of urinary bladder membranes: (1) hyaluronidase; (2) adrenal cortical extract (10% alcoholic, Upjohn Co.); alcoholic solutions of cholesterol, testosterone, estrone, desoxycorticosterone acetate,<sup>†</sup> Equilenin,<sup>†</sup> and Equilin;<sup>†</sup> (3) alarm reaction induced by colchicine. The final concentration of alcohol in the steroid tests did not exceed 5  $\gamma$  of ethanol per ml. This concentration was found to have no effect on membrane permeability. All substances tested for effect on permeability were added to the solution in contact with the mucosal surface.

**Skeletal muscle membrane.** The gastrocnemius muscle of frogs of near weight was immersed in a bath of 50 ml distilled water or 50 ml distilled water-drug solution, immediately removed from the solution and weighed after the water was shaken off. The muscle was then returned to its constant temperature bath maintained at 0 to  $2^\circ\text{C}$ . Thirty minutes after immersion and each one-half hour thereafter for 5 hours, the muscles were weighed. In several instances a weighing was also taken at 24 to 48 hours. Seventy-five muscles were used in this study and the following compounds were tested: hyaluronidase; an antihistaminic, Neohetramine; and histamine diphosphate.

**Results. Lens membrane.** Incubation of frog and rabbit lens in distilled water at  $38^\circ\text{C}$  resulted in progressive turbidity of the super-

natant (Fig. 1, curve A). The lens capsule usually blistered in one hour and ruptured up to 6 hours later. Incubation in physiological salt solution, on the other hand, did not produce turbidity and did not injure the capsule (Fig. 1, curve C). Addition of 0.05% hyaluronidase produced a marked increase in the turbidity of the distilled water (Fig. 1, curve B) but had no effect on the appearance of the physiological salt solution (Fig. 1, curve D). Tests with anthrone reagent<sup>7</sup> revealed that the turbidity was not due to release of carbohydrate material. This reagent, however, does not detect hyaluronic acid or its breakdown products.

It could not be assumed from the turbidity that hyaluronidase affected the permeability

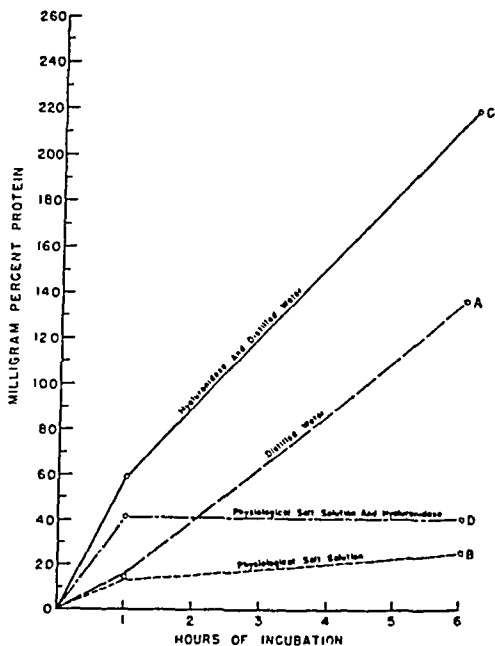


Fig. 2.

Leakage of proteins from rabbit lens exposed to hyaluronidase.

Curve A—Lens incubated at  $37^\circ\text{C}$  in distilled water. Capsule blistered in 1 hr.

Curve B—Lens incubated at  $37^\circ\text{C}$  in physiological salt sol.

Curve C—Lens incubated at  $37^\circ\text{C}$  in 0.05% hyaluronidase (assaying 9400 T.R.U. per mg N) in distilled water. Capsule blistered in 1 hr.

Curve D—Lens incubated at  $37^\circ\text{C}$  in 0.05% hyaluronidase (assaying 9400 T.R.U. per mg N) in physiological salt sol.

<sup>†</sup> We wish to thank the Schering Corp., Bloomfield, N. J., for the desoxycorticosterone acetate, and Ayerst, McKenna & Harrison Limited, Montreal, Canada, for the Equilenin and Equilin.

mosis through this bladder was more rapid in Tyrode's solution alone than through normal bladder membranes treated with hyaluronidase.

In order to determine whether an alarm reaction with the concomitant release of adrenal steroids would affect the permeability of membranes, 3 mg colchicine per kg were administered subcutaneously to rabbits. Two such membranes were prepared. Osmosis was completely inhibited through them even in the presence of hyaluronidase.<sup>†</sup> One ml of plasma from a rabbit in colchicine alarm reaction immediately inhibited osmosis and reversed the flow through a normal membrane.

**Striated muscle membrane.** Thirty gastrocnemius muscles immersed in distilled water gained an average of 82% of their original weight in 5 hours, 87% in 24 hours, and 78% at 48 hours (Fig. 4, curve A). Neohetramine inhibited the uptake of water so that at 5 hours there was an average increase to 62%, at 24 hours 39%, and at 48 hours only 31% (typical curve, Fig. 4, curve B), thus confirming the observations of Halpern<sup>11</sup> that antihistaminic drugs decrease the permeability of skeletal muscle immersed in distilled water. The uptake of water in gastrocnemius muscles immersed in a solution containing 0.1 to 0.2% histamine diphosphate was 73% of the original muscle weight at 5 hours, 103% at 24 hours, indicating a slight "edema" effect, and 98% at 48 hours (typical curve, Fig. 4, curve C). This concentration of histamine was sufficient to counteract the "anti-edema" effect of the lower concentrations of the anti-histaminic compound (Fig.

4, curve D). Neohetramine in a concentration of 1%, however, antagonized the effect of 0.1% histamine diphosphate.

Addition of hyaluronidase in amounts up to 1% in the distilled water solution alone or in the distilled water containing histamine or Neohetramine did not result in deviations from the usual behavior of the compound tested. The curves obtained in each instance could be superimposed on those shown in Fig. 4. Similar results were obtained with the rabbit soleus muscle incubated at 37°C.

The experiments with skeletal muscle are illustrative of our finding that hyaluronidase does not enhance penetration of drugs in isolated frog hearts and does not affect ultrafiltration through frog skin.

**Summary.** 1. Purified testicular hyaluronidase had no effect on the normal or altered permeability of skeletal muscle, cardiac muscle, or of isolated frog skin.

2. Hyaluronidase strikingly increased the permeability of the lens capsule and urinary bladder membranes, and abolished their semipermeable character.

3. The enhancing effect of hyaluronidase on osmosis through the bladder membrane was increased by desoxycorticosterone acetate.

4. Adrenal cortical extract, testosterone, and cholesterol abolished the enhancing effect of hyaluronidase. Estrone, Equilin, and Equilenin reversed the direction of flow through hyaluronidase treated membranes.

5. Hyaluronidase did not alter the complete inhibition of osmosis through the bladder membrane of rabbits which had been exposed to an alarming stimulus.

6. The products released *in vivo* during the alarm reaction rendered bladder membranes impermeable even in the presence of hyaluronidase.

<sup>11</sup> Halpern, B., personal communication. Published reference unavailable.

<sup>†</sup> Preliminary *in vitro* and *in vivo* experiments indicate that cortisone acetate is as effective as the alarm reaction.

TABLE II.  
Effect of Hyaluronidase, Steroids, and an Alarming Stimulus on the Permeability of Surviving Membranes.

	No. of expts.	Avg rise at 360 min. (mm)	Range (mm)	Median (mm)
Tyrode's sol.	5	39	38- 40	39
Hyaluronidase	5	160	159-161	160
Adrenal cortical extract + hyaluronidase	2	74	74- 75	
Estrone	1	20		
Estrone + hyaluronidase	1	13		
Desoxycorticosterone acetate	1	104		
Desoxycorticosterone acetate + hyaluronidase	1	150		
Equilenin + hyaluronidase	1	3		
Equilin + hyaluronidase	1	5		
Colchicine <i>in vivo</i>	1	0		
Colchicine <i>in vivo</i> + hyaluronidase <i>in vitro</i>	1	0		
Plasma from rabbit in alarm reaction	1	13		

after the first hour decreased the rate of osmosis, and adding it again at the third hour reestablished it. This alternation could be demonstrated for the first 6 hours.

Ten  $\gamma$  of either adrenal cortical extract, testosterone, or cholesterol per ml inhibited the passage of water through the membrane immersed in hyaluronidase solution. This observation resembles the *in vivo* inhibiting effect of adrenal cortical extract on the spreading activity of hyaluronidase<sup>8</sup> and decreased absorption through the synovial membrane.<sup>9</sup> Ten  $\gamma$  of estrone per ml not only stopped osmosis through the hyaluronidase treated membrane, but reversed the flow (Curve D). Injection of estrone *in vivo* increased the resistance of the ground substance<sup>10</sup> and of synovial membranes<sup>9</sup> to hyaluronidase.

The results obtained with fresh membranes in oxygenated Tyrode's solution were identical with those just described and are listed in Table II. Further studies were carried out using an estrogenic type steroid. Twenty-four  $\gamma$  of Equilenin or 30  $\gamma$  of Equilin per ml reversed the flow of water through the hyaluronidase treated membrane in a manner similar to that of estrone. When the compounds were removed and replaced with a fresh solution of hyaluronidase and Tyrode's solution, osmosis

proceeded as it had before the steroids had been added indicating that the membranes were not injured.

Ten  $\gamma$  of desoxycorticosterone acetate per ml enhanced considerably the effect of hyaluronidase on osmosis. To further study the action of this steroid, a rabbit was given 35 mg desoxycorticosterone acetate in oil intramuscularly once a week for two weeks. Os-

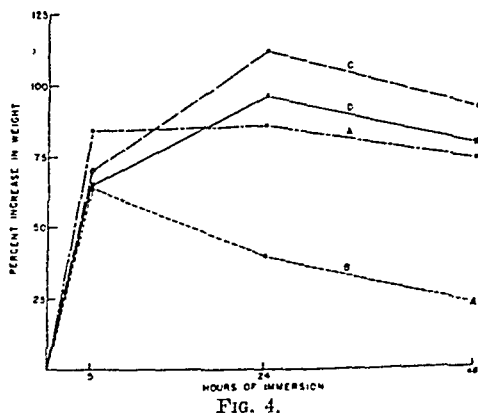


FIG. 4.

Influence of antihistaminics and histamine on lyophilized frog gastrocnemius muscle (immersed in distilled water at 2°C).

Curve A—Distilled water.

Curve B—0.1% neohetramine hydrochloride in distilled water. (1% neohetramine hydrochloride causes the muscle to decrease below the original weight).

Curve C—0.1% to 0.2% histamine diphosphate in distilled water.

Curve D—0.1% neohetramine hydrochloride plus 0.1% to 0.2% histamine diphosphate in distilled water. (1% neohetramine counteracts 0.1% histamine diphosphate).

<sup>8</sup> Opsahl, J., White, A., and Duran-Reynals, F., *Ann. N. Y. Acad. Sci.*, in press.

<sup>9</sup> Seifter, J., Baeder, D. H., and Begany, A. J., *Proc. Soc. Exp. Biol. and Med.*, 1949, **72**, in press.

<sup>10</sup> Sprunt, D. H., and McDermann, S., *Endocrinology*, 1940, **27**, 893.

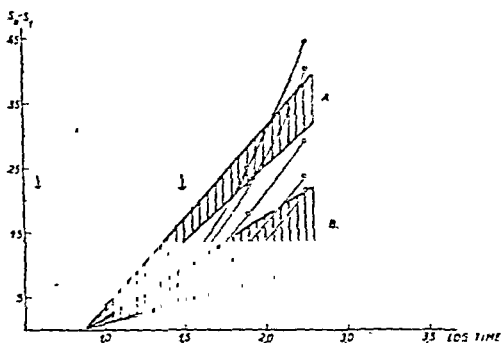


FIG. 1.

Growth of the methyl cholanthrene tumor of rats in a Carrel culture flask. Growth index:  $S_n - S_1$ .  $S_n$  is the measured growth at different times and  $S_1$  is the growth of the first day (before addition of the fluid phase). The shaded area A represents the control culture (embryonic extract plus Ringer's solution in fluid phase). The shaded area B shows the cultures where the liquid phase has been added twice (embryonic extract plus heparin 25 mg%). The solid lines show the cultures where the fluid phase has been added once (embryonic extract plus heparin 25 mg%). The time of growth is given on a logarithmic scale. Arrows show the changes of the fluid phases.

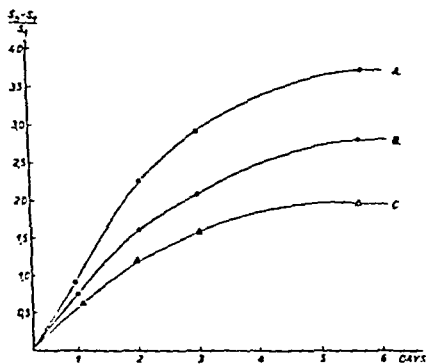


FIG. 2.

The growth of the methyl cholanthrene tumor of rats in Carrel culture flasks. Growth index:  $\frac{S_n - S_1}{S_1}$

( $S_n$  is the measured growth at different times, and  $S_1$  is the growth of the first day).

- A. Embryonic extract plus Ringer's sol.
- B. Embryonic extract plus sodium heparinate (40 mg%).
- C. Embryonic extract plus sodium salt of agar acid (40 mg%).

Before the fluid phase was added, the entire culture with the growth area was measured ( $S_1$ ). The growth area of the cultures ( $S_n$ ) were then determined every day. Data can be obtained from Fig. 1 and 2.

Fig. 1 illustrates the growth of those cultures which were treated with sodium heparinate (0.2 mg per flask). In the experiments, controls and experimental cultures are included. The fluid phase always contains the same concentration of growth promoting, water soluble, thermolabile substances of embryonic (12 days old chicken embryos) tissue-extract. To this fluid medium was added the test substances. The time is given logarithmically and indicates that the growth of the control cultures can be represented by a straight line from which the growth of the experimental cultures deviates markedly. When heparin is added once to the fluid phase an obvious suppression of the growth is observed; but this suppression is later compensated. This compensation shows itself by a marked increase in growth, in some cases even greater than that of the controls.

The heparin fluid phase in another series of cultures was changed twice. Under these conditions the growth was uniformly suppressed during the entire course of the experiment as shown by Fig. 1. Similar to the control cultures, the cultures repeatedly treated with heparin show a linear growth.

In an additional experiment, the growth of the cultures during continuous addition of sodium heparinate (40 mg % in every flask) and the sodium salt of agar acid was studied. Fig. 2 is a graphic representation of the relative growth of the cultures. In this case, the time scale is not logarithmical. It can be seen from the figure that the growth of the experimental cultures is considerably less than that of the controls. The most marked suppression of the growth was obtained when the agar salt was used, but the sodium heparinate also gives a similar suppression of growth. Using standard methods we have not been able to observe any difference in the morphology of the cells in the control and the cells in the experimental cultures.

*In vivo.* As our experiments have shown that the growth of tumor cells is suppressed by sodium heparinate and the sodium salt of agar acid, it seemed interesting to us to investigate whether the cellular growth could also be suppressed *in vivo*. Attempting to answer this question we have inoculated intraperi-



# Effect of Sulfomucopolysaccharides on Growth of Tumor Tissue.\* (17355)

A. BALAZS AND HJ. HOLMGREN (Introduced by A. Fischer.)

From the Department of Experimental Histology, Karolinska Institutet, Stockholm, Sweden.

Fischer<sup>1</sup> found that injury regeneration in tissue cultures is partially or completely suppressed by heparin. By treating transplants of Flexner-Jobling rat carcinoma with heparin, Goerner<sup>2</sup> could suppress its growth. Zakerzewski<sup>3-6</sup> made a systematic investigation of the influence of heparin on the growth of different tissues *in vitro*. He found that the growth of normal embryonic tissue was suppressed by heparin and that the same thing was true with Jensen sarcoma. He assumes that the growth-suppressing effect of heparin depends on an antiprothrombin action. Zakerzewski has also investigated the growth-suppressing effect of heparin on Jensen sarcoma in rats and on polymorphocellular sarcoma in mice. He could show that less tumor growth and less necrosis resulted from intravenous treatment. Fischer,<sup>7</sup> using pure heparin preparations, later showed that the suppressing effect of heparin on blood-coagulation and tissue growth disappeared after boiling with hydrochloric acid for a short time.

Balazs<sup>8</sup> has shown that other sulfonated mucopolysaccharides (the sodium salt of agar acid and the calcium salt of chondroitinsulfuric acid) as well as heparin suppress the growth of embryonic tissue *in vitro*. This suppressing effect depends on negative groups that are part of the polysaccharide. He could also show that there was a parallelism be-

tween the growth-suppressing effect and the number of negative groups per molecule. Only the undifferentiated tissues were found to be sensitive to the substances in question.

It is not only the growth of proliferated tissues from higher animal species that is suppressed by heparin. Fischer and Nyström<sup>9</sup> showed that the growth of yeast cells was also suppressed by heparin. Heilbrunn and Wilson<sup>10</sup> were recently able to demonstrate that heparin suppressed the mitosis of Chaetopterus eggs after fertilization. This action is reversible and has no degenerating effect on the eggs. They assume that heparin prevents the mitotic gelation.

**Experimental. *In vitro*.** Our *in vitro* experiments were performed on tumor cells from methyl cholanthrene tumors of the rat. All of the experiments were performed on cultures in Carrel flasks using a 2 phase system. (D 5). The cultures were placed in chick plasma without embryonic extract. In all the experiments the fluid phase of the control cultures has consisted of embryonic extract and physiological saline. Instead of the physiological saline, equivalent amounts of sodium heparinate (0.4 mg per flask) were added to the tissue cultures in one series, and an equivalent amount of the sodium salt of agar acid in another series. In preparing the sodium heparinate we used the powdered form having 13.35% moisture: S, 11.47% and N, 2.00%. This preparation we have gratefully received from Professor E. Jorpes. The sodium salt of agar acid preparation was made according to Hoffman and Gortner from the commercial agar. The preparations were dissolved in physiological saline solution (Ringer), and the pH was controlled between 6.7 and 7.2. In both cases the substances in question were added to cultures 24 hours old.

\* This work was aided by the Wenner-Gren Foundation, Sweden.

<sup>1</sup> Fischer, A., *Firchows Arch.*, 1930, **94**, 279.

<sup>2</sup> Goerner, A., *J. Lab. Clin. Med.*, 1930, **16**, 369.

<sup>3</sup> Zakerzewski, Z., *Z. f. Krebsforsch.*, 1932, **36**, 513.

<sup>4</sup> Zakerzewski, Z., *Bull. Internat. de l'Acad. Pol. Sci. et Lettre Cl. Med.*, 1932, 238.

<sup>5</sup> Zakerzewski, Z., *Arch. f. exp. Zellforsch.*, 1932, **13**, 152.

<sup>6</sup> Zakerzewski, Z., *Klin. Woch.*, 1932, **11**, 113 and 158.

<sup>7</sup> Fischer, A., *Protoplasma*, 1936, **26**, 344.

<sup>8</sup> Balazs, A., in press.

<sup>9</sup> Fischer, A., and Nyström, P., *Biochem. Z.*, 1933, **262**, 364.

<sup>10</sup> Heilbrunn, L. V., and Wilson, W. L., *Proc. Soc. Exp. Biol. and Med.*, 1949, **60**, 179.

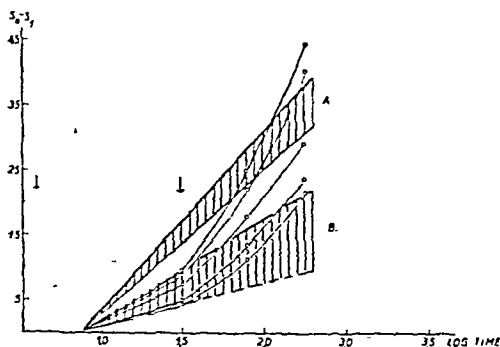


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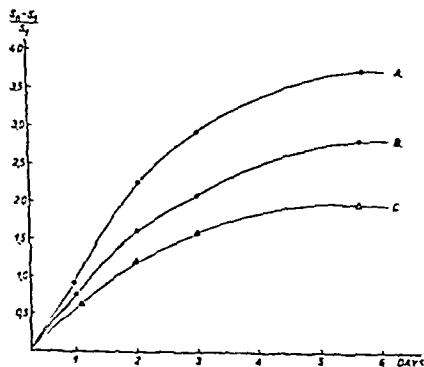


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The growth of the methyl cholanthrene tumor of rats in Carrel culture flasks. Growth index:  $\frac{S_n - S_1}{S_1}$

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The heparin fluid phase in another series of cultures was changed twice. Under these conditions the growth was uniformly suppressed during the entire course of the experiment as shown by Fig. 1. Similar to the control cultures, the cultures repeatedly treated with heparin show a linear growth.

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*In vivo.* As our experiments have shown that the growth of tumor cells is suppressed by sodium heparinate and the sodium salt of agar acid, it seemed interesting to us to investigate whether the cellular growth could also be suppressed *in vivo*. Attempting to answer this question we have inoculated intraperi-

toneally suspensions of cells from a solid Ehrlich's carcinoma into 300 white male mice (20-25 g) in 3 experiments.

In the first experiment the animals were given sodium heparinate together with the tumor cells intraperitoneally. In another series the animals were treated intraperitoneally with sodium heparinate for a few days after the tumor had been inoculated. In both these experiments it was found that the experimental animals started to die a few days later than the control animals. However, this difference was not persistent and the mortality curves gradually coincide. In a third experiment we have given a more consistent treatment. One hundred fifty mice were divided into 3 groups of 50 each. All were inoculated with an equal amount of tumor cell suspension (1.5 million cells in 0.1 ml physiological saline). At the same time 50 animals were given 0.3 mg sodium heparinate and 50 animals 0.3 mg of the sodium salt of agar acid subcutaneously. The third group served as a control. The experimental animals were treated daily with the same quantity of sodium heparinate and sodium salt of agar acid. The results obtained are seen from Fig. 3. The mortality of each of the series is calculated. It can be seen from the figure that the lines which represent the mortality of the 3 groups run parallel, which suggests that the difference between the groups is constant during the time of the experiment. At the end of the experiment, 11 days after the inoculation, 82% of the control animals, 72% of the heparinate treated animals, and 66% of the animals treated with agar extract had died. The experiment, it seems to us, indicates that there is a real difference in mortality between the control and experimental animals. As the groups are relatively large this difference could hardly be due to mere chance but is by all probability due to the treatment.

**Discussion.** Our results seem to confirm completely the statement of Zakerzewski that heparin suppresses the growth of tumor tissues *in vitro*. In addition to that we have shown that the sodium salt of agar acid has the same effect. The question arises how the effect of these sulfomucopolysaccharides is

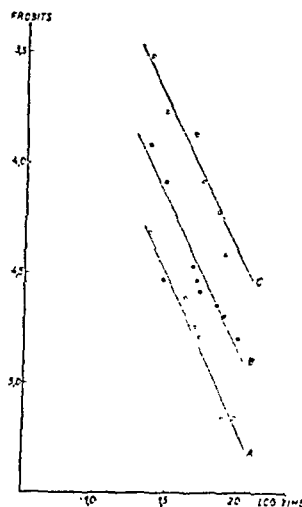


FIG. 3.

Dosage-mortality curve of ascites tumor of mice. Probits units are plotted against the logarithm of the time.

A. Control animals.

B. A total of 3 mg sodium heparinate injected subcutaneously during 9 days.

C. A total of 3 mg of the sodium salt of the agar acid injected subcutaneously during 9 days.

All the animals in the three groups have been given 1.5 million Ehrlich's carcinoma ascites cells intraperitoneally. The dosage-mortality curve has been calculated according to Bliss, C. L., *Quart. J. Pharmacol.*, 1938, 11, 192.

brought about *in vitro*. From this point of view there are two possibilities: either the sulfomucopolysaccharides used have a neutralizing effect on the growth promoting substance present in the embryonic extract and the original tissue, or they act directly on the growing cell. Whether or not the sulfomucopolysaccharides in the latter case suppress mitosis and migration, we cannot decide. The effect seems to be specific as shown by Balazs.<sup>6</sup> He found that the heart tissue from chick embryos pulsates faster and longer in a substrate containing sodium heparinate than in the control cultures containing embryonic extract. Using a suitable concentration of sodium heparinate, the growth could be completely suppressed in the former case.

Concerning the experiments *in vivo* it seems clear to us that death was delayed by treatment with sodium heparinate and the sodium salt of agar acid. We have not been able to prevent the death of the animals; but one must consider the fact that we are dealing

with a form of tumor which under these experimental conditions in 12 to 15 days produces a 100% mortality in mice. How the treatment has influenced the tumor cells is, of course, hard to tell. Three possibilities seem acceptable:

1. The sulfomucopolysaccharides used have a neutralizing effect on the toxic products derived from the tumor cells.

2. The sulfomucopolysaccharides exercise a general resistance-increasing effect on the tissues of the organism.

3. The sulfomucopolysaccharides in question exercise a direct growth-suppressing effect on the tumor cells.

Naturally, we have not been able to decide how the substances in question act; we only wanted to suggest these possibilities. It is of interest to note that Cramer and Simpson<sup>11</sup> and Holmgren and Wohlfart<sup>12</sup> have shown that in methyl cholanthrene tumors in mice and rats, great masses of mast cells can appear. Cramer and Simpson point out that the mast cells in the skin increase in number before the tumors are developed, and that resistant mice show an ample number of mast cells in the skin. They further point out that the reaction of the mast cell is involved in a defensive process directed against the

development of skin cancer. They also point out that this is of interest as the mast cells contain heparin (Holmgren and Wilander<sup>13</sup>), Jorpes, Holmgren, and Wilander.<sup>14</sup> It is also of interest that hyaluronic acid and other mucopolysaccharides are widely distributed in different tissues. Balazs and Holmgren<sup>15</sup> have shown that sulfomucopolysaccharides appear in injured tissue where they with all probability have a suppressing effect on the growth of the regenerating tissue.

*Summary.* Sulfomucopolysaccharides (sodium heparinate and the sodium salt of agar acid) suppress the growth of cells from methyl cholanthrene tumors in the rat *in vitro*. To ascertain whether the substances in question had a similar effect on growing tissues *in vivo* mice were inoculated intraperitoneally with the suspension of cells from Ehrlich's carcinoma, after which they were treated in various ways with sodium heparinate and with sodium salt of agar acid. It was found that the mortality of the control animals was greater than that of the treated animals. The results and various possible explanations are discussed.

<sup>13</sup> Holmgren, H. J. and Wilander, O., *Ztschr. f. mikr. anat. Forsch.*, 1937, **42**, 242.

<sup>14</sup> Jorpes, E., Holmgren, H. J., and Wilander, O., *Ztschr. f. mikr. anat. Forsch.*, 1937, **42**, 279.

<sup>15</sup> Balazs, A., and Holmgren, H. J., *Cell. Res.*, 1949, in press.

<sup>11</sup> Cramer, W., and Simpson, W. L., *Cancer Res.*, 1944, **4**, 601.

<sup>12</sup> Holmgren, H. J., and Wohlfart, G., *Cancer Res.*, 1947, **7**, 686.

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### Detection of Mumps Virus in Mice Sacrificed at Different Periods of Time after Injection. (17357)

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During the past 2 years we have experimented with the cultivation of mumps virus in embryonated chicken eggs, and have prepared inactivated virus vaccine for laboratory use and trial in human subjects.<sup>1</sup>

In the course of a portion of this work, we

have recently attempted to infect (looking for both apparent and inapparent infections) several of the smaller laboratory animals. To date, we have not been able to bring about any such infections or produce definite gross or microscopic lesions. We have used "raw" periembryonic egg fluid virus, and also such virus after concentration by the alcohol

<sup>1</sup> Muntz, H. M., Powell, H. M., and Culbertson, C. G., *J. Lab. and Clin. Med.*, 1949, **34**, 199.

TABLE I.  
Complement Fixation Tests of Periembrionic Fluids from Eggs Injected with Mouse Tissue Dilutions for Assay of Mumps Virus. Mice sacrificed 1 hour after injection of virus.

Mouse No.	Route inj.	Mouse organ	Dilution: 1 part in	Complement fixation titer of egg fluids					
				1:2	1:4	1:8	1:16	1:32	1:64
1	ip	Brain	10	—	—	—	—	—	—
			100	—	—	—	—	—	—
2	ip		10	++	—	—	—	—	—
			100	—	—	—	—	—	—
3	in		10	—	—	—	—	—	—
			100	—	—	—	—	—	—
4	in		10	—	—	—	—	—	—
			100	—	—	—	—	—	—
1	ip	Liver	10	++++	++++	++++	++	+	—
			100	++++	++++	—	—	—	—
2	ip		10	—	—	—	—	—	—
			100	—	—	—	—	—	—
3	in		10	+++	—	—	—	—	—
			100	—	—	—	—	—	—
4	in		10	++	—	—	—	—	—
			100	—	—	—	—	—	—
1	ip	Spleen	10	++++	++++	++++	++	—	—
			100	++++	++	—	—	—	—
2	ip		10	++++	++++	++++	++++	+	—
			100	++++	+++	+	—	—	—
3	in		10	—	—	—	—	—	—
			100	—	—	—	—	—	—
4	in		10	—	—	—	—	—	—
			100	—	—	—	—	—	—
1	ip	Lung	10	++++	++++	+++	±	—	—
			100	++++	++++	++++	++	—	—
2	ip		10	++++	++++	++++	+	—	—
			100	++++	+	—	—	—	—
3	in		10	—	—	—	—	—	—
			100	—	—	—	—	—	—
4	in		10	not tested		—	—	—	—
			100	+++	+	—	—	—	—
1	ip	Testes	10	—	—	—	—	—	—
			100	—	—	—	—	—	—
2	ip		10	—	—	—	—	—	—
			100	—	—	—	—	—	—
3	in		10	—	—	—	—	—	—
			100	—	—	—	—	—	—
4	in		10	—	—	—	—	—	—
			100	—	—	—	—	—	—

Legend: ip indicates intraperitoneally; in indicates intranasally.

method. Our negative results are in conformity with those of others who used mainly "raw" (*i.e.* non concentrated) virus. In the course of these tests, however, we have determined the titers of mumps virus in various organs and tissues at different times after injection of active virus, and the results of such tests showing appearance and disappearance of mumps virus in mice seem of sufficient interest to report.

Two groups of Swiss mice were injected

with Enders strain<sup>2</sup> of mumps virus in periembrionic fluid. This virus had a 4+ complement fixing (C.F.) titer of 1:16.<sup>3</sup> One group of mice received doses of 1.0 cc of this virus intraperitoneally. The other group of mice received doses of 0.05 cc of this virus intranasally. Pairs of mice from each group were

<sup>2</sup> Virus and information obtained from Dr. J. F. Enders, Boston, Mass.

<sup>3</sup> Bengtson, I. A., *Public Health Rep.* 1944, 59, 402.

sacrificed at 1 hour, 24, 48, 72 and 96 hours and at 2 and 3 weeks after injection. Dilutions of the homogenized organs obtained at autopsy were injected into 7 day embryonated eggs, and after proper incubation the periembrionic fluids from these eggs were tested for the presence of mumps virus by the C.F. test.

The results of the first test of mouse organs and tissues removed one hour after injection of mumps virus are shown in Table I. It is observed that spleen, lung and liver contained considerable virus. A trace of virus was found in the brain, while none was found in the testes. Although further negative results are not tabulated here, we found the virus neither in the pancreas, heart muscle, nor blood.

In these experiments, the mice which received intranasal virus got only one-twentieth the amount of virus given intraperitoneally. This intranasal dose however was sufficient to give rise to demonstrable titers of virus in the liver and lung one hour after injection.

Twenty-four hours after injection of mumps virus into mice we found, at autopsy, only

traces of virus in the spleen, lung, liver and brain. As before, no virus was found in the testes, pancreas, heart muscle, and blood.

No mumps virus was found in the organs and tissues heretofore examined in mice sacrificed at 2, 3, and 4 days, and at 2 and 3 weeks after injection of virus. Negative results after these longer periods of time indicate that inapparent infection does not occur.

*Summary.* Mumps virus administered to mice intraperitoneally or intranasally appears within an hour in the lungs, liver, spleen, and to a lesser extent in the brain (in the latter case after intraperitoneal injection only.) Sojourn of viable virus in organs where found is short. Testes, pancreas, heart muscle, and blood were found not to contain virus at any time. Mice used in these tests of mumps virus did not exhibit symptoms of toxicity at all comparable to mice injected at different times in the past with influenza virus, although these 2 viruses are somewhat similar otherwise.

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## Production of Acute Experimental Circulatory Failure by Graded Pulmonary Artery Constriction.\* (17358)

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Because of the practical and technical limitations of studying congestive heart failure in human subjects, numerous attempts have been made to produce heart failure in animals employing many different methods. Various drugs have been administered, including chloroform, ethyl alcohol, potassium chloride, chloral hydrate, and diphtheria toxin,<sup>1</sup> posterior pituitary extracts,<sup>2</sup> and quinidine,<sup>3</sup> in an effort to depress the myocardium. In ad-

dition, irritants have been injected into the pericardium<sup>4</sup> and myocardium;<sup>5</sup> the ventricular wall has been severely cauterized,<sup>6</sup> the coronary arteries ligated,<sup>7,8</sup> and the myocardial capillaries plugged with foreign sub-

<sup>2</sup> Nolasco, J. B., and Kohrman, R., *Proc. Soc. Exp. Biol. and Med.*, 1947, 66, 108.

<sup>3</sup> Reiss, R. A., and DiPalma, J. R., *Am. J. Physiol.*, 1948, 155, 336.

<sup>4</sup> Armstrong, T. G., *Quart. J. Exp. Physiol.*, 1940, 30, 263.

<sup>5</sup> Luisada, A., *Medicine*, 1940, 19, 475.

<sup>6</sup> Starr, I., Jeffers, W. A., and Meade, R. H., *Am. Heart J.*, 1943, 26, 291.

<sup>7</sup> Orías, O., *Am. J. Physiol.*, 1932, 100, 629.

<sup>8</sup> Gross, L., Mendlowitz, M., and Schauer, G., *Proc. Soc. Exp. Biol. and Med.*, 1936, 35, 446.

\* This research was supported by a grant from the Life Insurance Research Foundation.

<sup>†</sup> U. S. Public Health Service Research Fellow.

<sup>‡</sup> Sarah Welt Fellow.

<sup>1</sup> Fahr, G., and Buchler, M. S., *Am. Heart J.*, 1943, 25, 211.

stances.<sup>9</sup> In general, there have been two serious drawbacks to these methods: 1) the difficulty in controlling the severity of the experimental procedures, and 2) concomitant undesirable side effects, such as induction of arrhythmias and toxic actions on other organs of the body.

Since a reduced cardiac output—variously occasioned—is probably the initiating factor leading to the typical signs of congestive heart failure, the prerequisite for any technic attempting to produce experimental circulatory failure is a reduction of the output of the heart without lowering the blood pressure to shock levels. Therefore, we have employed a graded pulmonary artery stenosis as an experimental approach to the problem. This procedure possesses the following virtues: the cardiac output may be reduced significantly without concurrent marked changes in arterial pressure; a strain is placed on the right ventricle; any desired gradation of severity may be applied rapidly and easily; and finally, the procedure is readily reversible.

Previous workers who successfully produced experimental pulmonic stenosis were interested in its relation to pulmonary embolism<sup>10</sup> or in the cardiac dynamics of such a lesion,<sup>11</sup> and only incidental attention was directed to the effects on the peripheral circulation. Furthermore, there is only a narrow range of constriction which will give changes simulating clinical heart failure. If the constriction is too mild, right atrial and aortic pressures remain unaltered, and the only observable result is some increase in the right intraventricular pressure during systole. On the other hand, if the degree of constriction exceeds the optimum, an uncompensated drop in cardiac output occurs with a decrease in arterial blood pressure to shock levels.

Since we plan to utilize this technique in the future to determine the primary effects of reduction of cardiac output upon renal func-

tion, it seemed advisable to supplement our knowledge of the dynamic cardiovascular changes which are produced by increasing the resistance to right ventricular ejection. In addition, an attempt was made to elucidate the mechanism by which the central venous pressure becomes elevated during this procedure.

**Methods.** Mongrel dogs weighing between 8 and 16 kg were anesthetized with morphine followed by intravenous injection of sodium barbital (ca. 200 mg/kg). The chest was opened through the midline and respiration was maintained artificially. The pulmonary artery was dissected free near its origin, and was constricted by means of a strong cord noose attached to a screw device, permitting fine gradations of circular constriction. In each of the 13 experiments which were successfully performed, right atrial and aortic pressures were optically registered by means of modified Gregg manometers. In 5 of these experiments, right intraventricular pressures were also recorded, and in 5 others the pressures in the pulmonary artery distal to the point of constriction were recorded. A calibration was made at the end of each record in relation to a fixed base-line.

**Results.** No alterations in right atrial or aortic pressures could be detected until the pulmonary artery was constricted to a certain critical degree, estimated by previous workers<sup>10</sup> to be about 60% of occlusion. Pressure pulses registered from the aorta and right atrium during progressive, gradual constriction of the pulmonary artery are presented in Fig. 1 and 2. For reasons outlined by Wiggers,<sup>12</sup> right atrial pressures were measured at 3 points in all curves:

1. A point—the maximum point attained during atrial systole;
2. Z point—just prior to closure of the A-V valves; and
3. V point—just prior to opening of the A-V valves. These three points are indicated on the right atrial curve of record A in Fig. 1.

Using these criteria, records B and C of

<sup>9</sup> Roos, A., and Smith, J. R., *Am. J. Physiol.*, 1948, **153**, 558.

<sup>10</sup> Gibbon, J. H., Jr., Hopkinson, M., and Churchill, E. D., *J. Clin. Invest.*, 1932, **11**, 543.

<sup>11</sup> Fineberg, M. H., and Wiggers, C. J., *Am. Heart J.*, 1936, **11**, 255.

<sup>12</sup> Wiggers, C. J., *Physiology in Health and Disease*, 5th edition, Lea & Febiger, Philadelphia, 1949.

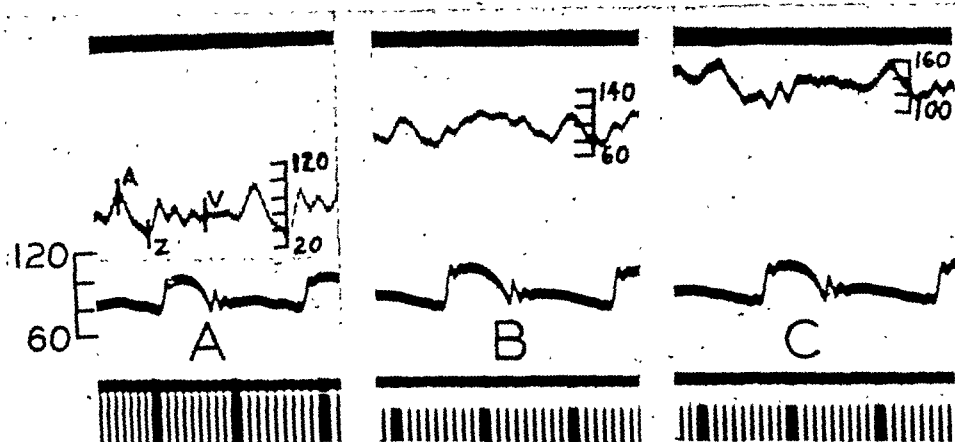


FIG. 1.

Right atrial (upper) and aortic (lower) pressure curves in experimental pulmonic stenosis. Record A—control; record B—minimal constriction beyond the critical degree; record C—slight additional constriction.

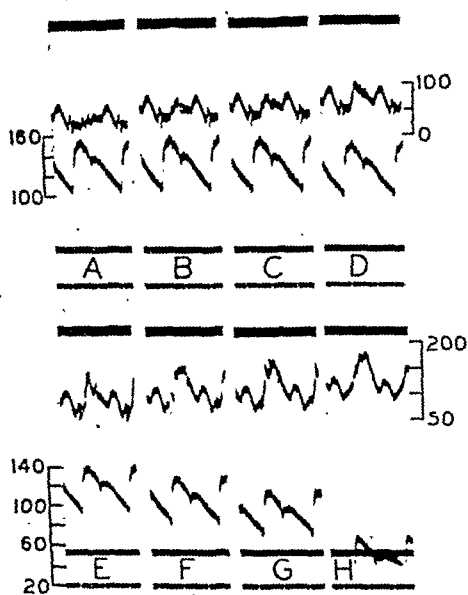


FIG. 2.

Right atrial (upper) and aortic (lower) pressure curves during control (record A) and progressively more severe pulmonic stenosis (records B through H). Discussion in text.

Fig. 1 reveal that a two-stage mild constriction of the pulmonary artery caused a relatively large elevation of the right atrial pressure as compared to control record A, with no significant alteration in the aortic pressure. This rise in right atrial pressure is present

at all points measured, but is most pronounced at the V point, amounting to an increment of 46 mm water in B, and of 83 mm water in C. The aortic systolic pressure remained within 5 mm Hg and the diastolic within 1 mm Hg of the control levels, the larger pulse pressure being due to a slight slowing of the heart. Such records show that lesions causing increased resistance to right ventricular discharge can effect an increase in central venous pressure without alteration of the arterial pressure and without graphic evidence that left ventricular discharge has been decreased.

We next studied an entire series of progressively severe, graded constrictions of the pulmonary artery in order to ascertain whether immediately recognizable criteria could be established which enabled one to determine that a significant reduction in cardiac output had been produced without lowering the arterial pressure to shock levels. The effects of such a sequence of graded constrictions are presented in Fig. 2.

Minimal constriction of the pulmonary artery beyond the critical point has elicited a 35 mm water elevation of right atrial pressure at the V point in record B and a further increase to 40 mm water in C, as compared to control record A, with no significant alteration in the aortic pressure. The first significant decrease in the aortic pressure is



present in record D, falling to 149/102 mm Hg, as compared to the control value of 155/110 mm Hg in record A. This slight drop in aortic pressure is accompanied by an additional rise in right atrial pressure of 55 mm water above control. This then is the first definite indication that the output of the left ventricle has been diminished.

With further compression of the pulmonary artery, the changes in right atrial and aortic pressures become more pronounced, as observed in records E through H. Even in record G, the aortic pressure is still within normal limits (116/80 mm Hg), while the central venous pressure has risen to 123 mm water above control levels, measured at the V point. Very slight additional constriction then produced a fall in aortic pressure to shock levels (record H). Throughout the entire series of records, no significant change in heart rate is present.

The circulatory conditions represented in records D to G simulate the cardinal features of clinical congestive heart failure, namely elevation of central venous pressure, reduction of cardiac output, and an arterial pressure within normal limits. We have been able to maintain these conditions for four hours or more, requiring only minimal adjustments of the clamp. Therefore, we shall attempt to reproduce these stages during our anticipated studies on the effects of reduced cardiac output upon renal function. The conditions existing in record H would, of course, be unsatisfactory since the arterial pressure is within shock levels. Stages B and C also would be unsatisfactory, since minute changes in cardiac output which might be present would probably be within the limits of error of any method for measuring the cardiac output (e.g. Fick principle).

A more detailed perusal of Fig. 2 reveals additional information which is not directly pertinent, but which is of some importance. It is evident in the right atrial curves registered during relatively severe stenosis of the pulmonary artery (records E through H) that a steep rise in slope occurs during ventricular systole, reaching a peak near the end of ventricular systole. These curves simulate

closely those described by Little during experimental tricuspid regurgitation,<sup>13</sup> and were recorded at a time when the central venous pressure was very high and the right ventricle was markedly dilated. Preliminary tests utilizing the injection of a small amount of concentrated dye (T1824) into the right ventricle tended to confirm the presence of tricuspid insufficiency. Within 3 to 5 seconds after injection during severe pulmonic stenosis, the concentration of dye in the right auricle and right ventricle were almost identical, and the amount of dye recovered from both sites was very large.

To further supplement our knowledge of the altered circulatory dynamics of pulmonic stenosis, pressures were recorded in several experiments from the pulmonary artery distal to the point of constriction during progressive compression of the pulmonary artery. As illustrated in Fig. 3, slight constriction beyond the critical point elicited a vibratory irregularity representative of a systolic murmur, as seen in the pulmonary artery curves of records B and C. In record B, the aortic pressure was slightly depressed, although quite frequently a pulmonic murmur was observed before any alteration occurred in the aortic or right atrial pressures. Furthermore, in some experiments the pulmonary artery and aortic pressures were slightly elevated when constriction just sufficient to produce a murmur was applied. In the pulmonary artery curves of records C and D, a sharp peak is present at the very beginning of systole. This is analogous to the anacrotic incisura described by Katz, Ralli, and Cheer in experimental aortic stenosis.<sup>14</sup> More severe constriction eventually resulted in a marked fall of systolic, diastolic, and pulse pressures in the pulmonary artery, as seen in record D. With release of constriction, these pressure changes were reversed, often with a transitory elevation of pulmonary artery pressures above control levels.

Right intraventricular pressures were re-

<sup>13</sup> Little, R. C., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 602.

<sup>14</sup> Katz, L. N., Ralli, E. P., and Cheer, S., *J. Clin. Invest.*, 1928, **5**, 205.

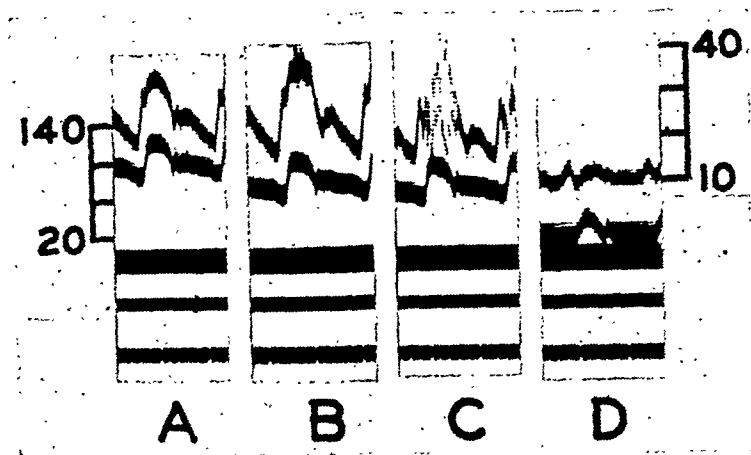


FIG. 3.

Pulmonary artery (upper) and aortic (lower) pressure curves during the control period (record A) and during gradually progressive pulmonic stenosis (records B through D).

recorded in several experiments during experimental pulmonic stenosis, and the findings were in accord with those reported by Fineberg and Wiggers.<sup>11</sup> To repeat these findings briefly, degrees of occlusion below about 60% elevated the systolic but not the diastolic pressures (initial tensions), while aortic and right atrial pressures were unaffected. This systolic pressure rise is believed to be a passive phenomenon, due solely to the increase in resistance, with no alteration in the force of ventricular contraction. Further degrees of occlusion eventually produced an elevation of initial tension as well as of systolic pressure. However, very soon after the rise in initial tension appeared, the systolic pressure began to diminish as the constriction progressed. This was considered by Fineberg and Wiggers to be evidence of myocardial failure, due to tremendous stretching of the myocardium, perhaps combined with diminished coronary flow, the result of decreased aortic pressure.

**Discussion.** As previously reported by Gibbon, Hopkinson, and Churchill,<sup>10</sup> progressive compression of the pulmonary artery beyond about 60% of occlusion produces a gradual elevation of central venous pressures and a fall in aortic pressures. With carefully graded constriction, we were usually able to reach a point where a moderate elevation of

right atrial pressure could be elicited with no significant alteration in the aortic pressure. With more severe degrees of occlusion, aortic pressures dropped, the right ventricle became markedly dilated, and curves were often obtained which were characteristic of tricuspid regurgitation. In attempting to explain the elevation of central venous pressures during this procedure, the marked venous distention observed during severe pulmonic stenosis indicates that the principal factor is an increase in the quantity of blood within the central veins. It is frequently stressed by

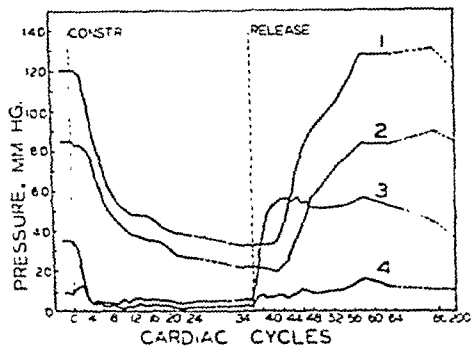


FIG. 4.

Plot showing the effects in successive beats of an abrupt occlusion and subsequent release of the main pulmonary artery on aortic systolic (1) and diastolic (2) and on pulmonary systolic (3) and diastolic (4) pressures, the latter two recorded distal to the occluding clamp. Discussion in text.

present-day investigators that it is dynamically impossible to produce an elevation of the venous pressure combined with a reduction in the cardiac output unless the blood volume is augmented. To prove this concept, many involved and complicated experimental procedures and physical models have been contrived.<sup>6,15,16</sup> Simple and decisive expedients, such as were used in this research, have largely been ignored. Our results demonstrate unequivocally that an increase in total blood volume is not an obligatory factor in causing an elevation of the central venous pressure, since extreme pressure variations can be produced within a few seconds, and can be maintained for many hours. The increase in the volume of blood contained within the central veins during our experiments is independent of any changes in total blood volume, and must be due to redistribution of the blood already present within the circulatory system.

Sufficient reduction of the lumen of the pulmonary artery acts primarily to diminish the output of the right ventricle. Initially, therefore, following constriction of the pulmonary artery there is a brief period in which the venous return to the heart exceeds the amount of blood pumped away by the right ventricle, resulting in a progressive increase in central venous pressure. Due to the decreased cardiac output, however, the venous return gradually diminishes, until it again becomes equal to the reduced cardiac output. The right atrial pressure then becomes re-stabilized, but at a higher level of pressure. It is within the relatively brief period during which there is an inequality between the cardiac output and venous return that a redistribution in the circulating blood volume has occurred. The problem resolves itself into an attempt to determine the source of the blood which contributes to the venous return over and above the suddenly diminished output of the right ventricle during this short period.

Fig. 4 presents graphic proof that a shift

of blood does occur between the pulmonic and systemic circulations during pulmonic stenosis. Following a sudden drastic occlusion of the pulmonary artery, the systolic and diastolic pressures in the pulmonary artery distal to the point of constriction dropped abruptly, and the pulse pressure was reduced almost immediately to minute values. However, systolic, diastolic, and pulse pressures in the aorta diminished very gradually over the course of about 30 beats. Thus, while the entry of blood into the pulmonary circuit was markedly reduced, blood still was extracted from the pulmonary vascular bed at almost the normal rate for a few cardiac cycles, and at a slowly diminishing rate for many more. When the constriction was suddenly released, the pulmonary arterial pressures rose almost immediately to super-normal values, while the aortic pressures recovered much more gradually, indicating a 'refilling' of the depleted pulmonary vessels to normal. Thus, our data favor the concept that the lungs serve an auxiliary function as a rather efficient blood reservoir interposed between the right and left ventricles. In addition to a shift of blood from the pulmonic to the systemic circuit, it is very possible that a transfer of blood occurs within the systemic circuit itself. This may take place from the arteries, capillaries, and small veins toward the central veins, or blood might be released from the various blood reservoirs located on the systemic side of the circulation, notably the spleen in the dog. This possibility deserves further study.

*Summary and conclusions.* An experimental attempt was made to simulate acutely the cardinal circulatory changes of congestive heart failure for the purpose of creating a preparation in which the incipient effects on renal function could later be studied.

Optically recorded pressure pulses from the aorta, pulmonary artery, and right atrium are presented, and show that judicious compression of the pulmonary artery can reduce the output of the left ventricle significantly without incurring a drastic fall of arterial pressure. These dynamic studies also revealed that such graded pulmonary artery constriction results in (a) a reduction of pulmonary sys-

<sup>15</sup> Starr, I., and Rawson, A. J., *Am. J. Med. Sc.*, 1940, 199, 27.

<sup>16</sup> Starr, I., *Am. J. Med. Sc.*, 1940, 199, 40.

tolic and diastolic pressures distal to the constriction, and the development of a systolic murmur, (b) a rise in maximal and initial tensions in the right ventricle, and (c) an elevation of right atrial pressures. In some instances, relative tricuspid insufficiency developed.

The elevation of central venous pressure

is obviously associated with translocation of a large volume of blood to the venous side of the circulation. Evidence is presented that a part of this transfer occurs from the pulmonary vascular bed, although it is admitted that a shift may also occur within the systemic circuit itself toward the central veins.

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### Cultivation of Poliomyelitis Virus in Cultures of Human Foreskin and Embryonic Tissues.\* (17359)

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Recently, the propagation *in vitro* of the Lansing strain of poliomyelitis virus in human embryonic tissues was reported and evidence presented that this virus is capable of multiplying in cells other than those of nervous origin.<sup>1</sup> These experiments have been continued and this agent now has been carried for a total period of 224 days through 13 serial cultures in which the tissue consisted of mixed human embryonic skin and muscle. This strain has also been maintained for 173 days in two lines of 11 serial cultures each and composed respectively of human embryonic intestine and brain.

Additional experiments described here in a preliminary manner are reported. Two objectives were in mind: (a) to determine whether the Lansing strain was capable of multiplying in completely differentiated non-nervous tissue as well as in embryonic tissue; (b) to determine whether the Brunhilde strain of poliomyelitis virus—a strain immunologically distinct from the Lansing group<sup>2</sup> and not

adaptable to rodents—could, like the Lansing strain, be cultivated in non-nervous human embryonic tissues.

*Propagation of the Lansing strain in human foreskin tissue.* As a source of completely differentiated non-nervous tissue fragments of human foreskin were employed. The use of this tissue was suggested by the report of Blank, Coriell, and Scott,<sup>3</sup> who explanted it on the chorioallantoic membrane according to the method of Goodpasture. The material was derived from patients between 4 and 11 years of age. Each prepuce was sufficient for the preparation of at least 8 cultures and before mincing was washed 2 or 3 times in nutrient fluid<sup>4</sup> containing 50 units each of streptomycin and penicillin per ml. The fluid phase of the cultures, which contained the same concentration of antibiotics was removed and replaced at intervals of 4 days. The original set of cultures was inoculated with 0.1 cc of a 10% suspension of pooled mouse brains infected with the Lansing strain. As inoculum for subcultures 0.1 ml of the pooled

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<sup>†</sup> Work done while a post doctorate Fellow of the United States Public Health Service.

<sup>‡</sup> Senior Fellow in Virus Diseases of the National Research Council.

<sup>1</sup> Enders, J. F., Weller, T. H., and Robbins, F. C., *Science*, 1949, 109, 85.

<sup>2</sup> Bodian, D., Morgan, I. M., and Howe, H. A., *Am. J. Hyg.*, 1949, 49, 234.

<sup>3</sup> Blank, H., Coriell, L. L., and Scott, T. F., *McNair, Proc. Soc. Exp. Biol. and Med.*, 1948, 69, 341.

<sup>4</sup> Weller, T. H., and Enders, J. F., *Proc. Soc. Exp. Biol. and Med.*, 1948, 69, 124.

TABLE I.  
Multiplication of Lansing Poliomyelitis Virus in Human Foreskin Tissue.

Culture set	No. of nutrient fluid changes prior to subculture	Day of incubation subculture done	Mouse LD <sub>50</sub> of pooled fluids used to inoculate subcultures	Calculated dilution of original inoculum at time of subculture
Original*	4	20	10-1.0	10-6
1st subculture	3	16	10-1.7	10-11
2nd "	3	16	10-1.2	10-16
3rd "	4	20	10-0.0	10-22
4th "	3	16	10-1.6	10-27
5th "	(in progress)			

\* The LD<sub>50</sub> of the infected mouse brain used in the original inoculum was 10-3.7; 0.1 cc of a 1:50 dilution of brain was employed and the calculated mouse LD<sub>50</sub> titer of the fluid phase immediately after inoculation was equal to 10-0.5.

centrifuged fluids removed on the 16th or 20th day of cultivation from the preceding set of cultures was used.

In the experiment summarized in Table I the virus was maintained for 88 days through 5 serial cultures of human foreskin. During this interval the fluid phase was removed and replaced 17 times. Pooled centrifuged supernatant fluids removed from the 4th culture series on the 20th day of cultivation when inoculated intracerebrally produced paralysis in a rhesus monkey. Histological changes consistent with a diagnosis of poliomyelitis were observed in the monkey's cord. Supernatant fluids removed from each serial culture produced paralysis in mice on intracerebral inoculation. Mice injected with fluids from uninoculated control cultures of tissues from the same sources remained well. It was calculated that during the 88-day period of cultivation the suspension of virus originally inoculated had undergone a dilution of at least 10<sup>-27</sup>. It would appear, therefore, that the Lansing strain is capable of multiplication in the presence of well differentiated skin and subcutaneous tissue and in the absence of intact nerve cells.

*Propagation of the Brunhilde strain in human embryonic tissues.* Two series of experiments were initiated utilizing the same technic. In one, the cultures consisted of mixed skin, muscle and connective tissue from the extremities of human embryos of 3 to 4 months gestation, and in the other of brain tissue from the same embryos. In each, the original set of cultures was inoculated with 0.1 cc of a 10% suspension of monkey cord infected with the

Brunhilde strain of poliomyelitis virus, supplied through the kindness of Dr. H. A. Howe. The skin and muscle series was subcultured three times to fresh tissues and the brain series twice.

Following intracerebral inoculation into rhesus monkeys of pooled centrifuged supernatant fluids of the skin-muscle series on the 12th and 21st days, respectively, from the second and third subcultures, muscular weakness of the lower extremities was observed. Histologic examination of the cords from these animals revealed changes consistent with infection by the virus of poliomyelitis. Fluids from the second subculture of the series prepared with brain tissue also produced paralysis on intracerebral inoculation into a rhesus monkey. No illness resulted after intracerebral inoculation of a portion of the same pooled supernatant fluids, from the 3rd subculture into Swiss white mice.

These findings indicate that the Brunhilde strain had been maintained for 73 days in the skin-muscle cultures and 39 days in those of brain. It was calculated that during the longer period the original inoculum of virus had been diluted 10<sup>-22</sup> times as a result of subculture and changes in the fluid phase.

According to Bodian<sup>5</sup> the usual titer of monkey cord infected with this strain for the rhesus monkey is 10<sup>-6</sup>. From the calculated dilution of the primary inoculum of the tissue cultures, it may, therefore, be inferred that this strain, like the Lansing virus, also multiplies *in vitro* in the presence of non-nervous tissue. Whether or not it can be propagated

<sup>5</sup> Bodian, D., *Am. J. Hyg.*, 1949, **49**, 200.

indefinitely under these conditions must await further investigation.

We are grateful to members of the Staff of the Boston Lying-in Hospital and the Children's Hospital for supplying the tissues. We also acknowl-

edge with thanks the indispensable assistance in the experimental work of Marguerite Buckingham and Jeanette Levens.

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## Fluorocardiography (Electrokymography) During Normal Respiration. (17360)

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Previous studies<sup>1,2</sup> described an apparatus, utilizing our modification of the Henny-Boone method of elektokymography,<sup>3,4</sup> and the typical patterns of tracings recorded over the major cardiovascular and pulmonary structures. Fluorocardiography was suggested as more descriptive than previously employed names for the method.

In previous studies, the tracings were recorded during voluntary apnea in an intermediate phase of respiration. Comparative observations showed that the pulsations of the lung (and, to a lesser extent, those of the hilar shadows and the pulmonary artery) were greater during inspiration than in sustained expiration. For practical purposes, an intermediate phase was chosen; a few words of instruction usually sufficed to teach the patient to hold his breath without excessive inflation or deflation of the lungs. This procedure was followed without demonstrable disadvantage in our entire series of clinical cases during 1947 and 1948. On the other hand, it was found difficult to obtain reliable tracings in children and patients with chronic diseases of the lungs who were unable to control their respiration because of age or dyspnea. For this reason, an attempt was

made to overcome this difficulty by technical means.

*Technical aspects.* The problem was presented to Maurice B. Rappaport, E.E. He suggested that a filter may be introduced to minimize respiratory effects though with the introduction of some error.

Wandering of the base line caused by respiration may be reduced considerably by altering the electrical time constant of the apparatus. When this constant is shortened, there is a proportionally greater attenuation of the coarse as compared with the more rapid waves. Respiration is represented by extremely slow waves in the fluorocardiogram, far coarser than any of those caused by cardiac action. A suitable condenser, or filter, introduced between the fluorocardiograph and the recording galvanometer, may modify the time constant of the apparatus; the smaller the condenser, the lesser is the electrical constant. Condensers of various size, which may be introduced by means of a switch, permit selection of the optimum time constant, namely the minimum value producing a fluorocardiogram that does not wander off the paper. The reduction of the electrical time constant of the apparatus does introduce a certain degree of error in the fluorocardiogram which is dependent upon the amount of reduction. The error presents itself in two forms: one is a slight error in the phase (or actual time) of registration of the component waves; the other is a slight change in their configuration. However, there is no elimination of the essential component waves present

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<sup>1</sup> Luisada, A. A., Fleischer, F. G., and Rappaport, M. B., *Am. Heart J.*, 1948, 35, 336.

<sup>2</sup> Luisada, A. A., Fleischer, F. G., and Rappaport, M. B., *Am. Heart J.*, 1948, 35, 348.

<sup>3</sup> Henny, G. C., and Boone, B. R., *Am. J. Roentgen.*, 1945, 54, 217.

<sup>4</sup> Henny, G. C., Boone, B. R., and Chamberlain, W. E., *Am. J. Roentgen.*, 1947, 57, 409.

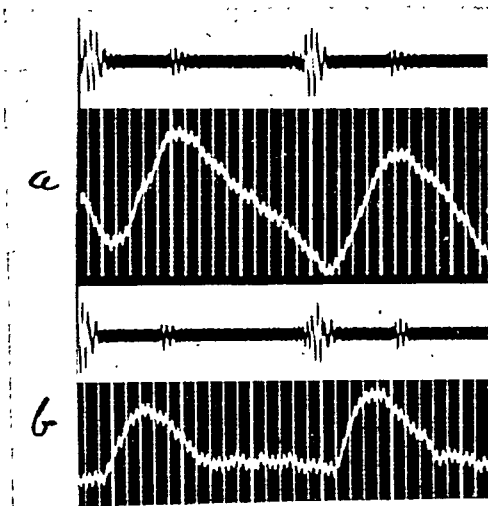


FIG. 1.

Pulsations of the pulmonary parenchyma (visible base of right lung) in a normal subject in apnea.

- (a) Conventional tracing (densogram).  
(b) Tracing obtained by adding an electrical filter.

under normal operating conditions. The filter used was arranged with an adjustable degree of low frequency damping. For optimal results, the degree of filtration was selected in each case to give a readable tracing with a minimal distortion of the fluorocardiogram.

Using this filter, it was possible to record the pulsations of the pulmonary vessels or any other cardiovascular structure during normal respiration with the following precautions:

(a) Whenever possible, the subject is instructed to breathe evenly and slowly, without sudden gasps.

(b) The roentgenologist observes the structure which is being studied and makes sure that it is not moving in and out of the slit during the respiratory movements.

In the practice, that degree of damping is chosen which will decrease the slow respiratory swing of the beam to a minimum with only slight diminution in amplitude of the waves caused by cardiac action. As shown in Fig. 1, a slight deformation of the waves is unavoidable. However, comparative studies on the height and initial rise of the pulse, made with the same degree of damping under otherwise similar conditions, proved the ob-

servations reliable. By means of the filter, several new studies were made possible.

*Results.* (A) *Fluorocardiogram of the child.* This fluorocardiogram, obtained by means of the above described filter, still presents occasional irregularities or wandering of the base line. This is due to the fact that children frequently exhibit a sharp beginning and end of inspiration with an abrupt change in phase; the filter cannot prevent slight wandering of the base line because of the irregularity and frequency of the respiratory waves. Nevertheless, clearly visible cardiac waves were obtained over all those cardiovascular and pulmonary structures which had been studied previously only in adults (Fig. 2). Now the field is open for the study of congenital heart diseases in childhood. In particular, the observation of a reduced amplitude of the pulsations of the pulmonary parenchyma is promising in the diagnosis of pulmonary stenosis.

(B) *Emphysema and pulmonary fibrosis with or without cor pulmonale.* In previous observations, we studied patients with chronic cor pulmonale who had only mild dyspnea and were able to hold the breath during the recording of fluorocardiograms. Other subjects with severe dyspnea yielded only poor tracings. By using the above described filter, two cases of chronic cor pulmonale with severe dyspnea were studied. As shown by Fig. 3, the details of the pulsations of the pulmonary and aortic knobs, as well as the pulsations of the lungs were recorded without difficulty.

(C) *Congestive failure.* Several cases of congestive failure have been studied by using this filter. These cases, which previously yielded only poor tracings, are currently studied and interesting observations are made concerning the lesser circulation.

(D) *Elimination of slow waves of cardiac origin.* In certain cases, a combination of cardiac disorders in an individual hinders a careful study of each. One patient observed by us had tricuspid insufficiency and auricular flutter. The study of the right auricle by means of the conventional apparatus revealed a systolic plateau similar to that previously described by us in the left auricle of patients

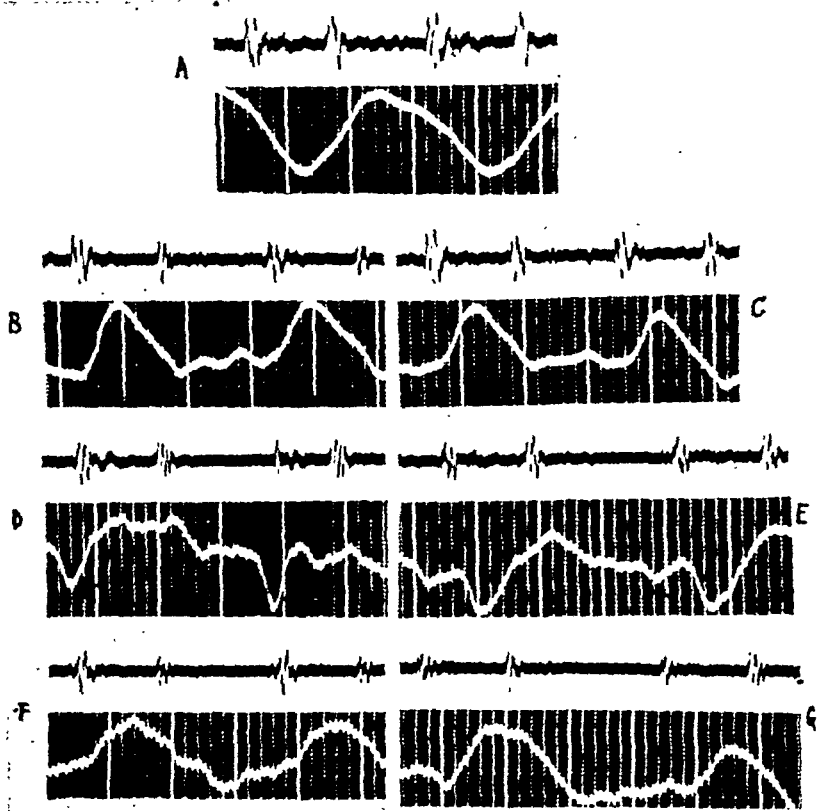


FIG. 2.

Tracings recorded on a 5-year-old normal boy during normal respiration with the use of the electrical filter.

A = Left ventricle; B = Pulmonary arch; C = Aortic arch; D = left auricle (right oblique); E = Right auricle; F = Right hilum; G = right lung; a—auricular wave of the auricular tracings.

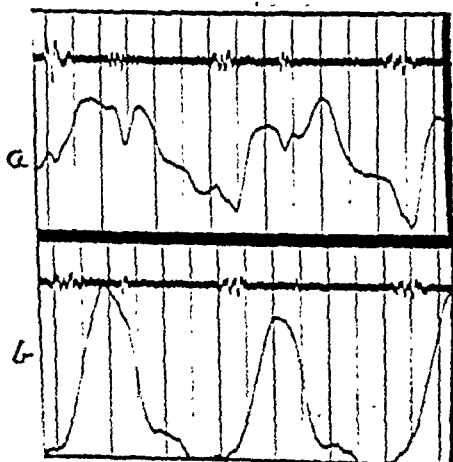


FIG. 3.

Tracings obtained during respiration by the use of the filter in a patient with severe pulmonary emphysema and dyspnea (border tracings).

(a) Pulmonary knob; (b) Aortic knob.

with mitral insufficiency and due to regurgitation of blood from the respective ventricle.<sup>5</sup> On the other hand, use of the filter permitted recognition of the rapid auricular rate of contraction (300 per minute) by eliminating part of the slow wave caused by the tricuspid insufficiency (Fig. 4).

**Summary.** (a) Fluorocardiography during normal respiration is possible by the use of an additional electrical filter, the physical

<sup>5</sup> Luisada, A. A., and Fleischner, F. G., *Am. J. Med.*, 1948, 4, 791.



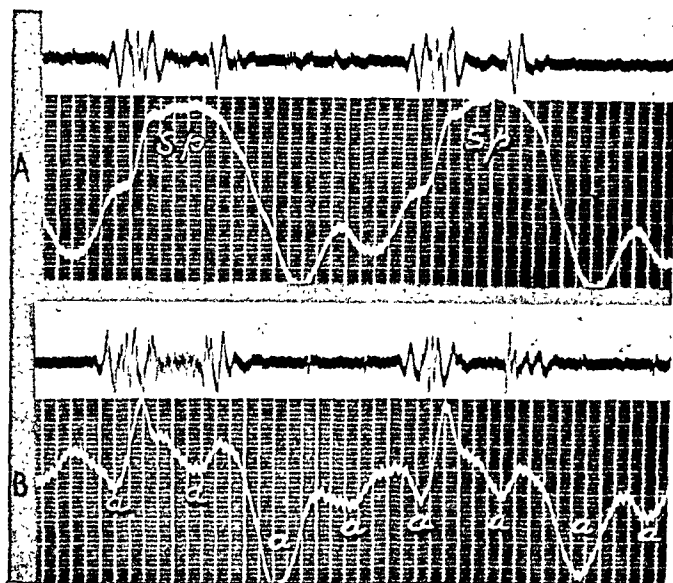


FIG. 4.

Tracings obtained during respiration in a patient with congestive failure, tricuspid insufficiency, and auricular flutter.

(A) Border tracing of right auricle (conventional tracing). A systolic plateau (SP) due to valvular insufficiency is apparent.

(B) Border tracing of right auricle (use of the filter). The rapid succession of auricular contractions (rate of 300) becomes apparent (a,a,a).

basis and principle of which are given.

(b) By using the filter, it is possible to record the fluorocardiogram of the child. This is of particular importance in congenital heart diseases.

(c) By using the filter, it is possible to study dyspneic cases of emphysema, pul-

monary fibrosis, or congestive failure.

(d) The use of the filter eliminates those slow waves of cardiac origin which may interfere with other rapid cardiac waves whose study is more important in particular cases.

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### Determination of Iodine-131 in Urine.\* (17361)

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In conjunction with research on the diagnostic and therapeutic value of radioactive iodine (8.0 day I-131) in thyroid disorders, a simple and accurate procedure for determining the amount of radioiodine excreted

is needed. Preparation of counting samples by evaporation of urine samples to dryness is a tedious and unpleasant procedure, and has also proved inaccurate, perhaps due partly to loss of some iodine by volatilization (possibly as volatile organic iodine compounds) and partly to variations in the geometrical character of the resulting solid samples.

\* The iodine 131 used in this investigation was supplied and obtained on allocation from the Atomic Energy Commission.

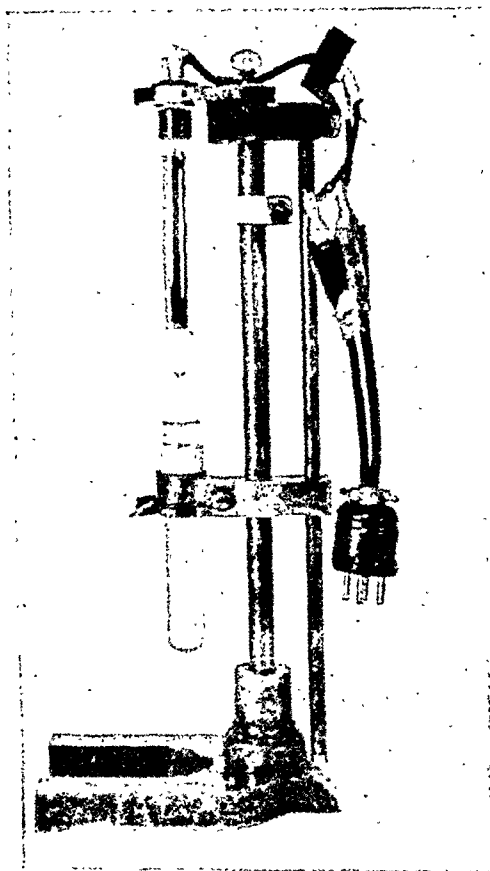


Fig. 1.

Shows the dipper counter (top) and sample tube (bottom) on microscope base.

A procedure for direct counting of the I-131 radiations in the urine by means of a dipping counter has been devised along lines similar to those reported by Solomon and Estes<sup>1</sup> for aqueous solution measurements. A thin-walled glass dipping counter (Radiation Counter Laboratories, Mark 1, Model 80) was mounted on a modified microscope base, as shown in Fig. 1. Samples of urine (sometimes diluted with water) were placed in test tubes whose length was exactly that of the glass portion of the counter tube (about 11 cm), and whose inside diameter was about 2 cm, 5 or 6 mm greater than the outside diameter of the counter tube. In the experi-

ments reported here, 8.5 ml of liquid proved optimum for covering the sensitive area of the counter when counter and sample tube were arranged concentrically.

*Experimental.* To reduce errors due to variations in geometry, all samples in a given series, including the standard urine, were counted in the same sample tube. Since the counter tubes showed some photosensitivity, the external surfaces of the sample tubes were blackened with India ink to exclude light.

The counter tube was used in conjunction with either a 64 scaler (Nuclear Instrument and Chemical Corp., Model 165) or a decimal scaler (Berkeley Scientific Company, Model 1000).

*Background Count.* Backgrounds were determined over a fifteen minute period with the sample tube containing 8.5 ml distilled water arranged in the experimental position.

*Standard Urine Count.* Standard urine samples were prepared by addition of a known volume of a previously standardized aqueous solution of I-131 to a known volume of normal urine. Samples of 8.5 ml of the resulting spiked urine were counted with the dipping counter, yielding values expressed as counts per minute per microcurie of I-131 per milliliter of urine. About ten microcuries per liter of urine makes a satisfactory standard (about 1000 counts per minute.)

*Experimental Samples.* Urine of the patients was collected in vessels containing 1 ml of KI solution (20 mg/ml), to decrease loss of iodine by volatilization. The specimens were then sampled (8.5 ml) for direct counting, or if too active (over 8000 counts per minute for 8.5 ml sample), were diluted with water to yield satisfactory counting samples. Counting periods were adjusted so as to yield a total of 5000 to 10,000 counts, so that standard errors were less than 2%. In addition, samples were counted in triplicate (3 separate 8.5 ml samples, placed successively in the same sample tube) to reduce errors due to variations in geometry and sampling. After each sample was counted, the sample tube and counter tube were washed twice with water, once with 6 N HNO<sub>3</sub>, and rinsed 3 times with distilled water. It was found advisable to

<sup>1</sup>Solomon, A. K., and Estes, H. D., *Rev. Sci. Instru.*, 1948, 19, 47.

check the background for a brief period after each count to determine whether decontamination was complete.

*Effect of Dilution of Urine.* Samples of a standard urine giving 195 counts per minute per 8.5 ml were diluted with varying amounts of water, and 8.5 ml samples of the dilutions were counted with the experimental apparatus to determine whether the diluted urine exhibited the same counting efficiency as the undiluted. Samples of total volume 8.5 ml were prepared, containing the following amounts of the standard urine: 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, and 8.5 ml.

*Results and Discussion.* Results of the experiment to determine effect of diluting the radioiodine-containing urine are shown in Fig. 2. It is apparent from the figure that addition of water to the urine does not affect the counting efficiency of the radiations, in spite of the variation in specific gravity. This is the anticipated result if the counts obtained are due largely to the gamma radiations from the I-131, as expected. The beta particles from this nuclide (0.60 Mev) should be essentially completely absorbed by a water layer of about 2 mm thickness.

Comparison of the experimental sample

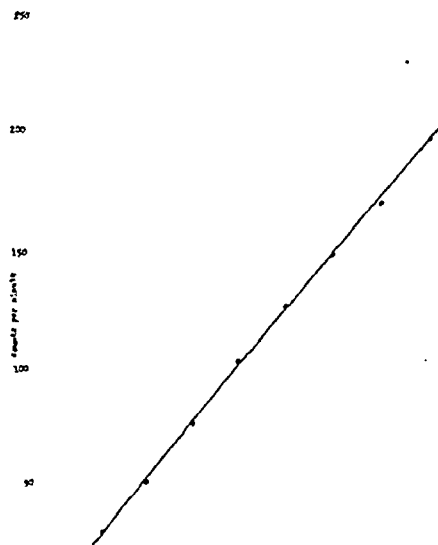
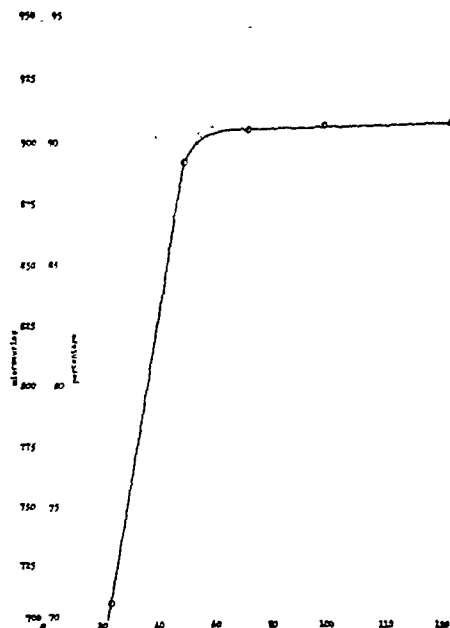


FIG. 2.  
Effect of dilution of urine.



Hours after administration.

FIG. 3.  
Urinary output of I-131. Tracer dose 100 microcuries.

counts (applying the dilution factor, if any) with the standard urine counts yields values in microcuries of I-131 per milliliter of experimental urine. Total output for the period over which the urine was collected is then obtained simply by multiplying this value by the total urine volume. Failure to collect the urine quantitatively is of course the greatest source of error in the measurements. Correction for radioactive decay since dosage may be applied either from the theoretical decay curve, or by counting the standard urine at the time of the experimental counts. Fig. 3 shows an actual series on a patient given 1000 microcuries of I-131. The curve indicates urinary output in microcuries and percentage, at daily intervals after administration.

Experiments now in progress are directed towards determination of the value of the urine measurements (*i.e.* rate of excretion of I-131) in diagnosis of thyroid disorders. It is believed that the determination of I-131 in urine is considerably more accurate than direct counting of the thyroid glands, which may vary considerably in size and shape, leading

to serious variations in geometry.

*Summary.* A method for the determination of Iodine-131 in urine is described. A dipping counter mounted on a modified microscope base using a blackened test tube with a

sample large enough to cover the sensitive area of the tube was used. Counting of gamma radiations is quite reproducible by this procedure.

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## Effect of Procaine Hydrochloride on Response of the Heart to Epinephrine During Cyclopropane Anaesthesia. (17362)

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While studying the production of ventricular fibrillation in dogs by the intravenous administration of epinephrine during cyclopropane anaesthesia, observations were made on the effect of procaine HCl on the cardiac irregularities. Its effect on the rate and rhythm of the heart during cyclopropane anaesthesia and its effect on the cardiac response to epinephrine were noted.

*Method.* A modification of Meek's technique was used.<sup>1</sup> Thirty minutes after premedication with 1.0 mg/kg morphine sulphate and 0.04 mg/kg atropine sulphate, 18 male and female dogs (4-10 kg in weight) were induced with a cyclopropane and oxygen mixture, intubated, and allowed a further 30 minutes to equilibrate with the cyclopropane-oxygen mixture administered by a to-and-fro carbon dioxide absorber. The mixture was adjusted to produce stage III, plane 3 anaesthesia. A total of 78 injections of epinephrine 0.01-0.03 mg/kg were made, taking 20 seconds for each injection, and with at least 20 minutes between injections. Procaine HCl 5-15 mg/kg in 5 cc saline was given in 50 seconds, 5 minutes before 32 of the epinephrine injections. On 7 occasions 50 mg/kg of procaine HCl were given and not followed by epinephrine. Electrocardiograms were recorded continuously during and following each injection. When ventricular fibrillation occurred, artificial respiration with 100% oxygen was instituted, thoracotomy performed,

and cardiac resuscitation attempted using manual massage and intracordial injections of procaine HCl 5-15 mg/kg.

*Effect on Cardiac Rate and Rhythm.* The cardiac rate was increased in all but two experiments when procaine HCl was given. (Table I). The degree of acceleration present 5 minutes after the injection increased with the dose employed (Table II). On 24 occasions irregularities were present at the time procaine HCl was injected, and with the exception of one sinus arrhythmia these changed to a regular sinus rhythm. (Table I).

*Modification of Cardiac Response to Epinephrine.* The response of the heart to epinephrine was notably different after the administration of 5-15 mg/kg of procaine HCl. Epinephrine was given 46 times without previous procaine HCl and 32 times following this agent. The occurrence of various rhythms following the epinephrine is shown in Table III. Calculation of "p" value<sup>2</sup> reveals a significant increase in the number of normal rhythms, a significant decrease in the number of cases with a sinus arrhythmia component in the rhythm, and no significant change in the incidence of either extrasystoles or of ventricular tachycardia.

An attempt was made to determine whether increased vagal tone was contributing to the electrocardiographic picture obtained following epinephrine injections. Signs taken to mean increased vagal tone were initial slowing, A-V block, and suppression of the sinus

<sup>1</sup> Meek, W. J., Hathaway, H. R., Orth, O. S., *J. Pharm. Exp. Therapy*, 1937, 61, 240.

<sup>2</sup> Mainland, D., *Can. J. Research*, 1946, E, 26, 1.

## CARDIAC IRREGULARITIES UNDER CYCLOPROPANE

TABLE I.  
Effects of Intravenous Procaine HCl (5-15 mg/kg) on Cardiac Rate and Rhythm Under Cyclopropane.

Rhythm before procaine HCl	No. of exp.	After procaine HCl	
		Reg. sinus rhythm	Acceleration
Regular sinus rhythm	8	8	7
Sinus arrhythmia	18	17	18
Heart block	2	2	2
Bigeminal rhythm	3	3	3
Irregular bradycardia	1	1	0
	32	31	30

TABLE II.  
Cardiac Acceleration with Procaine HCl.

Dose, mg/kg	No. of exp.	Avg rate when procaine given	Avg rate 5 min. after procaine
5	12	73.7	93.0
15	20	73.2	119.0
50	7	62.4	138.5

TABLE III.  
Rhythms Produced by Intravenous Epinephrine.

	No. of injects.	Rhythms observed			
		Reg. sinus	Vent. tachy.	Ex. systol	Sinus arr.
Epinephrine alone	46	2	17	17	9
Epinephrine after procaine HCl	32	9	10	10	0
"p" values		<0.01	>0.7	>0.7	<0.05

node with a slow nodal or ventricular rhythm. Evidence for an increase in vagal tone was absent in only ten of 46 records where epinephrine was given alone, but was absent in 29 of 32 records where epinephrine followed procaine HCl. The *p* value for this difference is <0.001.

Ventricular fibrillation occurred in 2 of 17 animals when epinephrine was administered alone, and in 3 of 12 when epinephrine was preceded by 5 mg/kg of procaine HCl. These 3 animals had all received the same dose of epinephrine in previous experiments with the onset of ventricular extrasystoles or ventricular tachycardia only. We were unable to restore a normal rhythm to the fibrillating ventricles using manual massage, artificial ventilation, and intracordial procaine HCl, 15 mg/kg.

*Comment.* Ectopic rhythms may arise following the depression of the normal pacemaker or as a result of impaired intracardiac

conduction. The administration of epinephrine may produce this effect reflexly through pressor-vagal reflexes with increase in vagal tone. Changes in vagal tone accompanying the phases of respiration are regarded as the cause of sinus arrhythmia. The increase in cardiac rate following procaine HCl, the absence of sinus arrhythmia, and the failure to demonstrate an increase in vagal tone following epinephrine injection all point to an anti-vagal action of procaine HCl in these experiments.

Burstein *et al.*<sup>3</sup> have reported that by the administration of procaine HCl (5 mg/kg) intravenously, cyclopropane-epinephrine induced ventricular fibrillation may be prevented from occurring, and that the fibrillating heart may be restored to a normal sinus rhythm. We feel that their own observations

<sup>3</sup> Burstein, C. L., Marangoni, B. A., DeGraft, A. C., and Rovenstine, E. A., *Anesthesiology*, 1940, 1, 167.

do not justify their conclusions, and our results are in disagreement on both points. Not only have we failed to show a protecting effect by procaine HCl, but like Stutzman *et al.*,<sup>4</sup> we have failed to revive the fibrillating heart by cardiac massage and the intracardial injection of 15 mg/kg of procaine HCl.

Allen *et al.*<sup>5</sup> have demonstrated a protecting action of procaine HCl in doses of 16 mg/kg against cyclopropane-epinephrine induced ventricular tachycardia, and feel that such protection is brought about by depression of the ventricular muscle. In their work the epinephrine and procaine HCl were given together, while in our experiments the procaine HCl preceded the epinephrine by 5 minutes. Our inability to duplicate their results may indicate that any such protection

of the myocardium is a transient one, particularly when larger doses of epinephrine are employed.

**Conclusions and Summary.** Intravenous procaine HCl increases cardiac rate and restores a regular sinus rhythm to those hearts showing irregularity during cyclopropane anaesthesia. This may account for the favorable clinical impressions on the use of procaine HCl.

A protecting action against cyclopropane-epinephrine induced ventricular tachycardia and fibrillation with doses of procaine HCl reasonable for prophylactic purposes during anaesthesia could not be demonstrated. In our opinion such protection has not yet been demonstrated by others.

Under the conditions of the experiment the predominating effect of procaine HCl given intravenously was an anti-vagal one.

<sup>4</sup> Stutzman, J. W., Allen, C. R., and Orth, O. S., *Anesthesiology*, 1945, 6, 57.

<sup>5</sup> Allen, C. R., Stutzman, J. W., Slocum, H. C., and Orth, O. S., *Anesthesiology*, 1941, 2, 503.

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### Response of the Syrian Hamster to Intradermal Injection of Modified Newcastle Disease Virus. (17363)

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The adaptation of the California strain (No. 11,914) of Newcastle disease virus to the Syrian hamster with 300 intracerebral serial passages and with 9 intranasal serial passages has been reported.<sup>1</sup> Various subcultures of hamster brain material, up to the 200th<sup>2</sup> intracerebral serial passage, were injected intradermally into 4-week-old hamsters (average weight 28 g). No evidence of Newcastle virus infection was noted, although hamsters injected intracerebrally with the

same brain suspension showed typical symptoms. No attempts were made to establish Newcastle disease by intradermal injection from the 200th through the 299th intracerebral passages.

The modified hamster virus was first successfully established intradermally by the inoculation of 0.1 cc of a 10% brain suspension of the 300th intracerebral passage in the abdominal region of four 4-week-old hamsters (average weight 28 g). Simultaneously 4 hamsters of the same age and weight were inoculated similarly with 0.1 cc of a 10% brain suspension of the 14th intranasal passage. Both groups of hamsters showed signs of involuntary motor reaction, evidence of salivation, and paralysis in 5 to 10 days.

<sup>1</sup> Reagan, R. L., Lillie, M. G., Smith, D. C., and Brueckner, A. L., *Proc. Soc. Exp. Biol. and Med.*, 1949, 71, 293.

<sup>2</sup> Reagan, R. L., Lillie, M. G., Hauser, J. E., and Brueckner, A. L., *Proc. Soc. Exp. Biol. and Med.*, 1945, 68, 293.

TABLE I.  
Intradermal Passage of a Strain of Hamster-Adapted Newcastle Virus.

Material used	Site of inoculation	Passage No.	No. animals inoculated	No. animals showing symptoms	No. days after inoculation symptoms appeared
0.1 cc of 10% suspension of 14th intranasal passage	Intradermally in abdominal area	1	4	4	5-7-7-10
		2	6	3	3-4-4
		3	6	2	5-6
		4	6	4	5-6-6-6
		5	6	4	5-6-6-7
		6	3	3	4-4-4
		7	6	3	4-5-5
		8	6	6	4-4-4-5-5-5
		9	4	4	4-5-5-5
		10	4	4	3-4-5-5
0.1 cc of 10% suspension of 300th intracerebral passage	Intradermally in abdominal area	1	4	4	5-6-6-10
		2	4	4	5-5-5-5
		3	4	4	5-6-6-6
		4	3	3	3-5-6
		5	4	4	3-5-5-6
		6	4	4	3-4-5-5

These symptoms are similar to those occurring in hamsters infected intracerebrally, although salivation has occurred regularly only in later passages. Brains were removed aseptically from the moribund hamsters of each series, ground with alundum, and diluted to a 10% suspension with physiological saline. One-tenth cc of this suspension from each series was injected intradermally into hamsters. By using this technic, the 300th intracerebral brain material was carried up to 6 passages intradermally, and the 14th nasal brain material was carried up to 10 passages intradermally, as shown in Table I. These subinoculations are being continued at the present time.

To determine the distribution of Newcastle virus in the affected hamsters of each series, blood, liver, spleen, kidneys, lungs, spinal cord, and brain were injected into embryonated eggs. Each organ was removed aseptically, ground with alundum and diluted to a 10% suspension with physiological saline. One-tenth cc of citrated blood and of each organ suspension was injected into the allantoic sac of each of six 8-day embryonated White Leghorn eggs. Because of possible bacterial contamination, the lungs, kidneys, liver, and spleen were treated with streptomycin in the ratio of 1 mg per cc of suspension and with penicillin in the ratio of 500 units per cc of suspension before egg inoculation.

Virus was isolated from the brain and spinal cord of hamsters of each series. Virus from the brain and cord of each series was completely neutralized by positive Newcastle chicken serum but it was not affected by normal chicken serum. Virus was not isolated in embryonated chicken eggs from the blood or other organs of either series.

Day-old chicks were injected intramuscularly with 0.2 cc of a 10% suspension of brain material from each series. All chicks showed symptoms of Newcastle disease from 3 to 10 days after injection. Six-week-old chickens were injected intramuscularly with the same amount of a 10% suspension of brain material from each series but showed no evidence of Newcastle disease.

*Discussion.* In the subinoculations up to the 200th intracerebral hamsters passage, hamsters were not infected intradermally. Upon trial of the 300th intracerebral passage and of the 14th intranasal passage, hamsters were infected intradermally, when brain material was used as the inoculum. The 300th intracerebral passage was carried through 6 subinoculations intradermally, and the 14th intranasal passage was carried 10 subinoculations intradermally.

With hamsters as the test animals, virus neutralization tests were conducted, using brain suspensions of the 3rd intradermal passage of the 300th intracerebral series, and

TABLE II.  
Titration of Hamster-Adapted Newcastle Virus in Hamsters.

Virus	Dilution					Titer
	10 <sup>-1</sup>	10 <sup>-2</sup>	10 <sup>-3</sup>	10 <sup>-4</sup>	10 <sup>-5</sup>	
Hamster brain 5th intradermal passage originally from 14th nasal passage	*4/4	4/4	4/4	0/4	0/4	10 <sup>-3.5</sup>
Hamster brain 3rd intradermal passage originally from 300th intracerebral passage	3/3	4/4	2/4	0/4	0/3	10 <sup>-3</sup>

\* Numerator denotes No. of deaths.  
Denominator denotes No. of animals inoculated.

the 5th intradermal passage of the 14th intranasal series. Specific immune chicken serum completely neutralized the intradermal brain virus from both series, whereas normal chicken serum had no effect.

The virus titrated  $10^{-3}$  in the first series (300th intracerebral) and  $10^{-3.5}$  in the second series (14th intranasal), according to the Reed-Muench calculation.<sup>3</sup> Details are given in Table II. Paralysis of the pharynx, as evidenced by excessive salivation with wetting of the mouth and chin, is now a distinct symptom in hamsters.

It has been observed in other modified forms of this virus that day-old chicks are susceptible, whereas older individuals are resistant to infection.

<sup>3</sup> Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

*Summary.* After the 300th intracerebral and the 14th intranasal hamster passage, Newcastle disease virus, California strain No. 11,914 can successfully infect hamsters by intradermal injection. The virus in the brain material from each series was passed intradermally in hamsters. Virus was isolated from the brain and cord of hamsters of both series upon inoculation of embryonated chicken eggs. The results of the neutralization tests confirm the virus to be that of Newcastle disease. Hamsters infected intracerebrally, intranasally, and intradermally show similar symptoms, such as involuntary motor reactions, excessive salivation from pharyngeal paralysis, general paralysis, prostration, and death.

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## Effect of Oxythiamine on the Growth of Chicks.\* (17364)

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Ithaca, N. Y.

Bergel and Todd<sup>1</sup> reported the synthesis

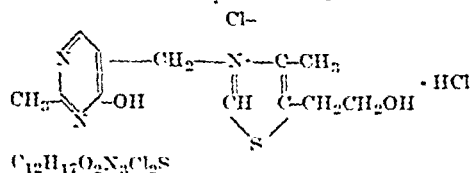
\* This work was undertaken in cooperation with the Office of Naval Research, Navy Department, Washington, D.C., and was aided by a grant to Cornell University by the Nutrition Foundation, Inc., New York.

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<sup>1</sup> Bergel, F., and Todd, A. R., *J. Chem. Soc.*, 1937, 1504.

of oxythiamine.<sup>‡</sup> They found that 1 mg of this compound possessed no antineuritic ac-

‡ The formula of oxythiamine is





tivity when assayed by the bradycardia method using the rat. Soodak and Cerecedo<sup>2</sup> prepared oxythiamine by deamination of thiamine with nitrous acid, and observed that the compound produced a marked toxic effect when fed to mice. The administration of 25 to 50  $\mu\text{g}$  of oxythiamine per day for a 2-week period resulted in the death of young mice maintained on a thiamine-low synthetic diet supplemented with 1  $\mu\text{g}$  of thiamine per day. In this report evidence is presented which shows that oxythiamine is also an active antagonist of thiamine in the nutrition of the chick.

*Experimental.* Four experiments were conducted with day-old White Leghorn cockerels, 10 or 15 per lot. They were housed in electrically heated battery brooders in a room in which the temperature was thermostatically controlled. All chicks were on wire mesh floors to prevent coprophagy. Feed and water were supplied *ad libitum*. The chicks were fed basal diet 653 described by Hill, Norris, and Heuser,<sup>3</sup> modified by adding 100  $\mu\text{g}$  of synthetic folic acid per 100 g of diet and by omitting thiamine. In the first 3 experiments crude casein was used instead of purified casein. In these experiments the basal diet contained between 20 and 25  $\mu\text{g}$  of thiamine per 100 g, as determined by the thiochrome method. The procedure used was that proposed by the Research Corporation Committee on the thiochrome method.<sup>4</sup> The basal diet fed in Experiment 4 in which purified casein was used contained 7  $\mu\text{g}$  of thiamine per 100 g. The chicks were weighed individually at weekly intervals.

*Exp. 1 and 2.* Two preliminary experiments were conducted to study the effects of graded levels of oxythiamine<sup>§</sup> on the growth of chicks to 4 weeks of age. Oxythiamine at

the levels of 0.5, 1, 2, and 8 mg was added to 125  $\mu\text{g}$  of thiamine per 100 g of diet. The results indicated that oxythiamine retarded growth at all levels used; the 8 mg level was so highly toxic that only one chick survived to 3 weeks of age. The chicks showed the typical thiamine deficiency symptoms of head retraction, convulsions and inability to stand. Two chicks with these severe symptoms were given 100  $\mu\text{g}$  of thiamine by injection, and within 2 hours they were on their feet and eating. The average 4-week weights of the chicks ranged from 289 g for those receiving 125  $\mu\text{g}$  of thiamine to 182 g for those receiving 125  $\mu\text{g}$  of thiamine plus 2 mg of oxythiamine per 100 g of diet.

*Exp. 3.* This experiment was designed to obtain a quantitative estimate of the effectiveness of oxythiamine as an antagonist of thiamine. The data from Exp. 1 and 2 indicated that the levels of oxythiamine to feed with approximately 125  $\mu\text{g}$  of thiamine per 100 g of diet for the best results were between 1 and 2 mg. Exp. 3 was conducted using an experimental diet containing 120  $\mu\text{g}$  instead of 125  $\mu\text{g}$  of thiamine per 100 g plus 3 levels of oxythiamine. The basal diet contained 5  $\mu\text{g}$  less thiamine per 100 g than the diet fed in Exp. 1 and 2. Eight levels of thiamine were included in order to establish a growth curve. The results of Exp. 3 are presented in Table I.

When oxythiamine was fed in addition to 120  $\mu\text{g}$  of thiamine a marked reduction in growth occurred. By plotting a curve for the effect of varying amounts of thiamine on growth, it was possible to determine the amount of thiamine that was counteracted by the different amounts of oxythiamine. An index was calculated by determining the ratio of the amount of inhibitor to the amount of vitamin antagonized by the inhibitor. The range of the 3 indices obtained in this experiment was 27-39.

*Exp. 4.* This experiment was designed to study the reversibility of this antagonism by additions of thiamine. The basal diet contained purified casein. Increasing levels of thiamine were added to a level of 2 mg of oxythiamine per 100 g of diet. This study was continued for a period of 4 weeks. The

<sup>2</sup> Soodak, M., and Cerecedo, L. R., *J. Am. Chem. Soc.*, 1944, **66**, 1988.

<sup>3</sup> Hill, F. W., Norris, L. C., and Heuser, G. F., *J. Nutrition*, 1944, **28**, 175.

<sup>4</sup> Hennessy, D. J., *Cereal Chemists' Bull.*, 1942, **2**, 1.

<sup>§</sup> The authors are indebted to Dr. Gustav J. Martin, The National Drug Company, Philadelphia, Pa., for the oxythiamine used in these experiments.

TABLE I.  
Inhibitory Action of Oxythiamine on Chick Growth.

Treatment, $\mu$ g thiamine	Avg wt		Index*
	3 wk, g	4 wk, g	
1. 45†	— (0)‡	— (0)	
2. 70	142 (4)	161 (4)	
3. 80	152 (9)	218 (7)	
4. 90	162 (12)	251 (10)	
5. 100	176 (14)	264 (13)	
6. 110	187 (15)	250 (15)	
7. 120	180 (15)	264 (15)	
8. 220	175 (15)	266 (14)	
9. 120 + 1 mg oxythiamine	179 (15)	233 (14)	27
10. 120 + 1.5 mg oxythiamine	136 (15)	191 (15)	34
11. 120 + 2 mg oxythiamine	118 (14)	157 (12)	39

\* Index is the ratio of amount of inhibitor to amount of vitamin inhibited.

† Crude casein basal diet contained 20  $\mu$ g thiamine per 100 g. The levels of thiamine indicate the total thiamine present per 100 g of diet.

‡ Numbers in parentheses represent the surviving chicks of original 15.

TABLE II.  
Reversal of Inhibitory Effect of Oxythiamine by Thiamine.

Treatment, thiamine	Avg wt		Index*
	3 wk, g	4 wk, g	
1. 62 $\mu$ g†	75 (1)‡	— (0)	
2. 72 $\mu$ g	99 (4)	86 (1)	
3. 82 $\mu$ g	128 (8)	126 (5)	
4. 92 $\mu$ g	140 (7)	168 (6)	
5. 102 $\mu$ g	147 (9)	194 (9)	
6. 112 $\mu$ g	177 (10)	230 (10)	
7. 122 $\mu$ g	185 (10)	259 (10)	
8. 1 mg	172 (10)	246 (10)	
9. 122 $\mu$ g + 2 mg oxythiamine	105 (9)	124 (9)	49
10. 147 $\mu$ g + 2 mg oxythiamine	116 (10)	153 (10)	34
11. 172 $\mu$ g + 2 mg oxythiamine	121 (10)	172 (9)	25
12. 1 mg + 2 mg oxythiamine	160 (10)	244 (9)	

\* Index is the ratio of amount of inhibitor to amount of vitamin inhibited.

† Purified casein basal diet contained 7  $\mu$ g thiamine per 100 g. The levels of thiamine indicate the total thiamine present per 100 g of diet.

‡ Numbers in parentheses represent the surviving chicks of original 10.

results of this experiment are presented in Table II.

The addition of 2 mg of oxythiamine to 122  $\mu$ g of thiamine caused a 135 g retardation in growth. However, as more thiamine was added to this level of oxythiamine growth was improved. Fifty  $\mu$ g of added thiamine resulted in a 48 g increase, while the addition of 1 mg of thiamine produced normal growth. It is possible that much less than 1 mg of thiamine would have given maximum growth. The inhibitory index for the three levels of thiamine ranged from (25-49).

At the end of the 4th week the chicks of lots 9, 10, and 11 of Exp. 4 were divided into 3 groups. Three chicks from each lot were

continued as controls, 3 received injections of 200  $\mu$ g of thiamine per day into the breast muscle, and 3 received 200  $\mu$ g of thiamine per day by stomach tube. These chicks were selected to give groups of equivalent weights. All chicks were continued on the diets they had received from the start of Exp. 4. Lot 7 receiving 122  $\mu$ g of thiamine was continued as the positive control group. The study was carried out for 12 days. The results are summarized in Table III.

The toxicity produced by oxythiamine can be at least partially corrected by additional thiamine. The data show that by injecting or giving thiamine orally a growth response was produced. Injection was somewhat more

TABLE III.  
Effect of Parenteral and Orally Administered Thiamine on Oxythiamine-Toxic Chicks.

Treatment	Avg initial wt,* g	Avg final wt,† g	Avg change in wt, g
1. Control	160‡	262	102
2. Injected§	153	304	151
3. Oral	153	290	137
4. Positive control¶	286	490	204

\* Weight at 4 wk of age.

† At end of 12-day experimental period.

‡ Avg of 8 chicks, rest are avg of 9 chicks.

§ 200 µg thiamine inj. per day into breast muscle.

|| 200 µg thiamine per day by stomach tube.

¶ Group 7 of Exp. 4 received 122 µg thiamine per 100 g of diet from hatching.

effective than oral administration. In a 12-day period the chicks receiving thiamine gained 35 to 49 g more than the controls, although as should be expected, their growth rate was less than that of the positive controls.

**Discussion.** Oxythiamine produced a thiamine deficiency state in chicks, which could be prevented, or cured to some extent, by administering increased amounts of thiamine. This antagonist was very effective as evidenced by the inhibitory index of 25-49. Although this index is an approximate value it is quite low and comparable to that obtained by Woolley and White<sup>5</sup> for the pyrithiamine: thiamine index in mice of 40. It has been shown by Woolley and White<sup>6</sup> that a low pyrithiamine:thiamine index is obtained with bacteria that require thiamine as the intact molecule. If a microorganism can utilize one or both moieties of the thiamine molecule, the index is 10 to 100 times greater. The chick requires the entire thiamine molecule, thus a low oxythiamine:thiamine ratio is to be expected.

Whether the oxythiamine-thiamine inhibition is a strictly competitive one is doubtful. In Table I it is noted that the chicks in lot 2 averaged 161 g at 4 weeks of age with 4 chicks surviving of the original 15, whereas those in lot 11 grew at the same rate, 157 g, but 12 chicks survived. The same effect is observed in Table II, where chicks receiving low levels of thiamine showed a greater mortality than chicks receiving both oxythiamine and a

higher level of thiamine, although the growth rates were similar. (Compare lots 3 and 9, 4 and 11). If simple competition were involved similar mortality would be expected in these lots of chicks.

The requirement of thiamine for White Leghorn cockerels on a purified casein diet is considerably greater than on a crude casein diet. On the purified casein diet the requirement for optimum growth to 4 weeks of age was between 102 and 112 µg of thiamine per 100 g. Whereas, on the crude casein diet the requirement was between 80 and 90 µg per 100 g. The thiamine values obtained by the thiochrome method on replicate samples of each diet were very consistent, although the concentration was low, 0.07 µg per g and 0.2 to 0.25 µg per g, respectively. The explanation for these different requirements is not known, although it may be that the purification process removed from casein some substance that affects the requirement of thiamine. The addition of liver and fish meal supplements to the purified diet produces a significant growth response.<sup>7</sup> It is possible that one of the unknown growth factors present in these materials and crude casein and not in purified casein to any great extent, may affect the requirement of thiamine.

**Summary.** Oxythiamine, the 4'-OH analogue of thiamine, has been shown to be an effective thiamine antagonist in the nutrition of the chick. By increasing the level of oxythiamine fed, the chick weight was decreased until typical thiamine-deficiency symptoms occurred and death ensued. The addition of

<sup>5</sup> Woolley, D. W., and White, A. G. C., *J. Biol. Chem.*, 1943, **149**, 285.

<sup>6</sup> Woolley, D. W., and White, A. G. C., *J. Exp. Med.*, 1943, **78**, 489.

<sup>7</sup> Unpublished data, Agricultural Experiment Station, Cornell University, Ithaca, N. Y.

large amounts of thiamine to the diet prevented the toxicity due to oxythiamine, and the parenteral and oral administration of thiamine to oxythiamine-toxic chicks tended to overcome the inhibition.

The chick, as well as the mouse, has been shown to be very sensitive to the addition of oxythiamine to the diet. It seems probable, therefore, that oxythiamine is an antagonist

of thiamine in the nutrition of all species that require this vitamin as an essential nutrient.

Evidence has been presented which indicates that the thiamine requirement of growing White Leghorn cockerels is greater in the absence of sufficient of the unidentified chick growth factors.

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### The Virostatic and Virucidal Action of $\alpha$ -Haloacylamides on Vaccinia Virus *in Vitro*. (17365)

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During the course of an investigation dealing with the possible antiviral effects of pyrimidine derivatives<sup>1</sup> it was found that amides of 5-aminouracil<sup>1</sup> inhibit the multiplication of vaccinia virus in the tissue culture system previously described.<sup>2</sup> Based on antagonisms demonstrable using *Lactobacillus casei*<sup>3</sup> it was predicted that the climax of this series would be reached with 5-chloroacetamidouracil or amides of 5-aminouracil with other acids having pK<sub>a</sub> values near that of chloroacetic acid. Actually the activity of chloroacetamidouracil was greater by a factor of 10 or more than the predicted value. Further investigation revealed a high degree of antiviral activity to be a function of all the chloro- and bromoacylamides and dichloroacetamides which were tested including chloroacetamide itself. This paper presents the data on the *in vitro* tests.

\* These studies were begun at Western Reserve University Medical School (1945) and continued at the Medical College of Virginia (1946-7).

<sup>1</sup> Thompson, R. L., Wilkin, M. L., Hitchings, G. H., Elion, G. B., Falco, E. A., and Russell, P. B., *Science*, 1949, 110, 454.

<sup>2</sup> Thompson, R. L., *J. Immunol.*, 1947, 55, 345.

<sup>3</sup> Hitchings, G. H., Elion, G. B., Falco, E. A., Russell, P. B., and Vander Werff, H., *Annals N. Y. Acad. Sci.*, in press.

*Experimental.* Screening tests with vaccinia virus in chick embryonic tissue culture were carried out as described previously.<sup>2</sup>

*Discussion.* It will be seen (Table I) that bromo- and chloroacylamides usually inhibit growth of the vaccinia virus. The inactivity of the fluoroacetamidouracil (Compound 14, Table I) suggests that an alkylation reaction may be involved in the activity.

When the high degree of activity of 5-chloroacetamidouracil was discovered attention was turned first to the embryonic tissue test system. It was a recognized weakness of the screening test that substances capable of poisoning the embryonic tissue undoubtedly would be expected to prevent the multiplication of the virus.<sup>2</sup> Thus known poisons, such as azide and cyanide, could be shown to inhibit viral multiplication.<sup>2</sup> On the other hand, 5-chloroacetamidouracil was no more toxic to mice than related substances of much lower antiviral potency. It is ineffective against the majority of bacteria, and in reasonable concentration fails to affect the respiration and glycolysis of tissue slices or the glycolysis of washed cell suspensions of *Lactobacillus casei*.<sup>1</sup>

<sup>1</sup> We are indebted to Doris Lorz for these determinations.

TABLE I.  
Virucidal Activity of Various Haloacylamides.\*

No.	Formula			Compound	Ref.	Conc.† mg per l	Increase in virus titer (logarithm)	
							Control	Treated
1.	$\text{ClCH}_2\text{CONH}_2$					100	2.03	-1.16
	<div><div><div><div>1</div><div>N</div></div><div>2</div><div><div><div>3</div><div>N</div></div><div>4</div></div><div>6</div></div></div> <div><div><div><div></div><div></div><div></div><div></div><div></div><div></div></div><div></div><div></div></div></div> <div><div><div></div><div></div><div></div><div></div><div></div><div></div></div><div></div><div></div></div> <div><div><div></div><div></div><div></div><div></div><div></div><div></div></div><div></div><div></div></div> <div><div><div></div><div></div><div></div><div></div><div></div><div></div></div><div></div><div></div></div> <div><div><div></div><div></div><div></div><div></div><div></div><div></div></div><div></div><div></div></div> <div><div><div></div><div></div><div></div><div></div><div></div><div></div></div><div></div><div></div></div> <div><div><div></div><div></div><div></div><div></div><div></div><div></div></div><div></div><div></div></div> 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\* Determined as described by Thompson.<sup>2</sup>

† The minimal effective concentration is given.

‡ We are indebted to Prof. Alex. R. Todd for this substance.

§ These hitherto undescribed compounds were prepared in The Wellcome Research Laboratories.

|| 1,3-Dimethyl derivatives.

5 Beckurts, H., and Frerichs, G., *Arch. Pharm.*, 1915, **253**, 233.6 Jacobz, W. A., and Heidelberger, M., *J. Am. Chem. Soc.*, 1917, **39**, 2420.7 Meyer, P. J., *Ber.*, 1875, **8**, 1153.8 Russell, P. B., Elion, G. B., and Hitchings, G. H., *J. Am. Chem. Soc.*, 1940, **71**, 474.

When the structure of chloromycetin (Chloramphenicol) (IV, Fig. 1) became known,<sup>4</sup> the relationship of this dichloroacetamide to such compounds as 5-chloroacetamidouracil (I, Fig. 1), 5-dichloroacetamidouracil

<sup>4</sup> Rebstock, M. C., Crooks, H. M., Jr., Controulis, J., and Bartz, Q. R., 115th Meeting Am. Chem. Soc., San Francisco, Calif., 1949. Abstracts 9K.

cil (II) and  $\alpha$ -chloro-*p*-nitroacetanilide (III) became apparent. The previous work suggests that the virucidal center of chloromycetin resides in the dichloroacetamide linkage and that all haloacylamides are potentially virucidal. It suggests the possibility that some of the simpler haloacylamides may find practical application in the sterilization of

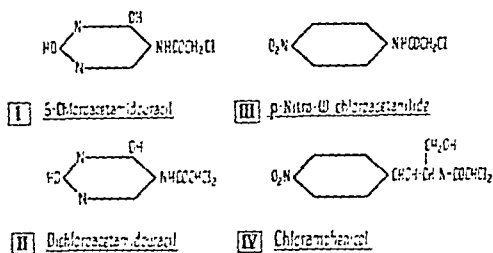


FIG. 1.

substances from the standpoint of viral contamination.

These findings suggest that the tissue culture system when properly controlled is capable of the demonstration of active virucidal chemical groupings. Virucidal substances dis-

covered in this way may be thought of as comparable to bactericidal substances discovered in the usual *in vitro* bacterial growth systems. The relationship between such substances and useful chemotherapeutic agents may be as uncertain in the one biological system as it is in the other.

**Summary.** Several chloro- and bromoacylamides were demonstrated to inhibit multiplication of the vaccinia virus in chick embryonic tissue. 5-Dichloroacetamidouracil was found to be inhibitory whereas 5-fluoroacetamidouracil was inactive.

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## Seasonal Variation in Human Fasting Blood Sugar Levels.\* (17366)

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From the Bureau of Human Nutrition and Home Economics, Agricultural Research Administration, U. S. Department of Agriculture, Washington, D.C.

During the course of a study on human subjects involving the effect of the breakfast meal on carbohydrate metabolism,<sup>1</sup> seasonal variation in fasting blood sugar levels was noted.

Work done in this laboratory over a 2 year period on 9 women gave a total of 170 fasting blood sugar determinations. When these values were averaged by months, the results shown in Fig. 1 were obtained. The fasting values during the 2 year period were within the range given by Peters and Van Slyke.<sup>2</sup> However, they showed marked seasonal differences. Blood sugar concentration rose progressively with the colder months of the year and were definitely higher in the winter than during the summer months.

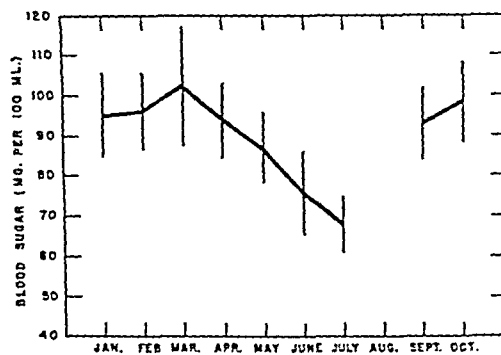


FIG. 1.

Average blood sugar values by month. Standard deviations indicated by vertical lines.

The subjects used in this study were 9 women laboratory workers ranging in age from 28 to 48 years. They were of average weight and height and moderately active. They were considered to be in good health although no physical or medical examinations were made.

Periodic blood counts and urine analyses were normal. At no time did any of the subjects participate in an experiment when suf-

\* This research was done as part of a project supported by an allotment made by the Secretary of Agriculture from Special Research Funds (Bankhead-Jones Act of June 29, 1935).

<sup>1</sup> Orent-Keiles, Elsa, and Hallman, Lois F., U.S.D.A. Circular, 1949, No. 827.

<sup>2</sup> Peters, J. P., and Van Slyke, D. D., Quantitative clinical chemistry, Vol. 2, Chap. III, Williams and Wilkins, 1946.

fering from a cold or similar indisposition. Neither were tests made during menstruation. By coincidence none of the subjects were smokers.

Samples of blood were taken after 15 hours' fasting and collected from a fingerprick into a paraffin well. Folin's micro method<sup>3</sup> was used for the glucose determinations, using 1.6% sodium carbonate in place of the sodium cyanide-sodium carbonate solution as recommended by Klendshoj and Hubbard.<sup>4</sup> Readings were made on a Beckman spectrophotometer at 5200 Å. All measurements and read-

ings throughout the study were done by the same analyst. Periodic standard sugar curves were made to check the solutions and the instrument. A search of the literature revealed only 2 observations<sup>5,6</sup> which suggest a relationship of season to concentration of blood sugar in human subjects.

The results obtained in the study reported here present the question whether these seasonal differences are due to variation in the quality or quantity of the food ingested or difference in the individual metabolism, or both, at different seasons of the year.

<sup>3</sup> Folin, O., and Svedberg, D., *J. Biol. Chem.*, 1930, **83**, 85.

<sup>4</sup> Klendshoj, Niels C., and Hubbard, Roger S., *J. Lab. Clin. Med.*, 1939-40, **25**, 1102.

<sup>5</sup> Johnson, Buford, J., *J. Comp. Psychol.*, 1922, **2**, 155.

<sup>6</sup> Strouse, S., *Arch. Int. Med.*, 1920, **26**, 758.

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## Hearing in Guinea Pigs Deficient in the Anti-Stiffness Factor.\* (17367)

ROSALIND WULZEN AND ALICE B. PLYMPTON.

*From the Department of Zoology, Oregon State College, Corvallis, Ore.*

This research was outlined to establish whether a diet deficient in the anti-stiffness factor<sup>1-5</sup> produced diminution in the hearing ability of guinea pigs. Responses of guinea pigs to sound are various. The most frequently observed reflex is the well-known ear-flick which, when well developed, is a folding back of the ear against the side of the head, but in diminished form is a mere quiver of the edge of the ear. This type of response was chosen as the most clear-cut obtainable to establish the hearing range of guinea pigs, although certain animals gave evidence by

jerking or movement of whiskers that they heard a little beyond the limits set by the ear-flick.

Stimuli were furnished by a General Electric audio-signal generator combined with a loud speaker and key (borrowed through the kindness of Prof. E. A. Yunker, Department of Physics, Oregon State College). Free response was obtained by placing the unconfined animal upon the table, closely facing the loud speaker. Each guinea pig was exposed to a series of signals from a frequency of zero to a frequency of 17,000 cycles per second. The range in pitch to which ear-flick response was given was found to be 150-16500 cycles per second. Fluctuations in electric current from time to time produced slight variations in response.

The 68 experimental animals used in this research had been fed a diet deficient in the anti-stiffness factor for at least 11 months before the testing started. This diet consisted of 18-20% skim milk powder in water, to which was added copper and iron salts and the following assortment of vitamins: The

\* Supported in part by a grant from General Research, Oregon State College.

<sup>1</sup> Wulzen, R., and Bahrs, A. M., *Physiol. Zoology*, 1936, **9**, 508.

<sup>2</sup> Wulzen, R., and Bahrs, A. M., *Am. J. Physiol.*, 1941, **133**, 500.

<sup>3</sup> van Wagtenonk, W. J., *J. Biol. Chem.*, 1944, **155**, 337.

<sup>4</sup> Simonsen, D. H., and van Wagtenonk, W. J., 1947, **170**, 239.

<sup>5</sup> Folkers, K., *Chem. and Physiol. of Growth*, 1949, 82-86.

salts and ascorbic acid, 10 mg per animal, were added to the morning feeding. The fat soluble and the water soluble vitamins were added alternately to the evening feeding, allowing 2 cc of the solutions for each animal.

Vitamin solutions		
Water soluble 1 liter	Thiamine hydrochloride	0.2 g
	Riboflavin	0.5
	Pyridoxine hydrochloride	0.1
	Nicotinic acid	1.0
	Calcium pantothenate	0.1
	Inositol	10.0
	p-Amino benzoic acid	2.0
	Choline hydrochloride	50.0
	Folic acid	0.003
	Biotin concentrate	3 drops
Fat soluble 1 liter	Beta carotene	20 cc
	Vioosterol	10 cc
	Alpha tocopherol	0.1 g
	2-CH <sub>3</sub> -1, 4-naphthaquinone	0.1 g

The 68 deficient animals were compared with 68 non-deficient stock guinea pigs which had been fed a diet of rolled barley, with abundant green feed daily. Both groups of animals were bedded in wheat straw and provided with iodized salt and water.

Both stock and deficient animals were subjected to frequent hearing tests over a period of 7 months. In both groups it was found that individuals differed from one another in hearing to some degree, but this difference involved almost exclusively the lowest extent of the hearing range, up to about 2000 cycles

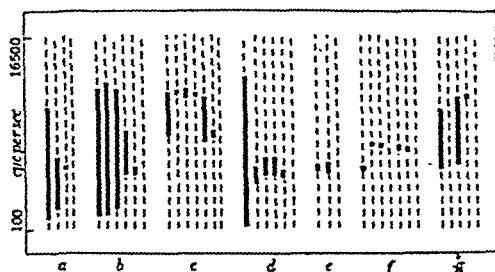


FIG. 2.

Seven individual cases of deficient guinea pigs showing recession of hearing during an experimental period. Length of individual lines indicates range in pitch of audio-signals, 100 to 16,500 cycles per second. Solid portion of line indicates actual hearing range of deficient animal on day tested. Each group of lines represents successive hearing responses of one animal. The decline of each animal to final deafness is shown by an overall decrease in length of solid lines. Final dotted line in each group shows entire absence of hearing response, which was proved by many subsequent tests to be permanent deafness.

Intervals in days between testing dates were as follows: (a) 30, 30, 23. (b) 30, 45, 23, 14, 8. (c) 30, 45, 10, 4, 3, 16. (d) 30, 45, 23, 10, 4. (e) 30, 45. (f) 30, 35, 10, 4, 3, 17. (g) 30, 35, 17, 16.

per second. The upper limit of hearing with apparently normal animals was very regularly somewhat above 10000 cycles per second.

The 68 non-deficient animals showed normal range of ear-flick response at each test over the 7-month period of observation, the most restricted range being 2,000-10,000 (Fig. 1, upper). Of the 68 deficient tested, 11 never gave ear-flick response at any time during the 7-month period. It should be noted that these animals had been on deficient diet for at least 11 months before the first test. By the end of the 7-month experimental period, 8 more guinea pigs had become deaf (gave no ear-flick response), making a total of 19 deaf animals (Fig. 1, lower). The advancing deafness of 7 of these individuals is illustrated in Fig. 2.

Statistical analysis was made of data on hearing ability of 56 stock animals and 64 deficient animals. The average ranges (highest frequency at which ear-flick was obtained less lowest frequency at which ear-flick was obtained) are given in Table 1. The analysis of variance shows that the animals which were fed with the same diet differed from one another in range of response to audio-signals ( $F = 34.75$  with 118 and 429 degrees of

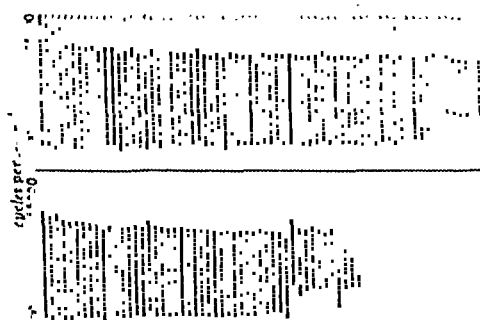


FIG. 1.

Upper series represents hearing range of 68 guinea pigs fed stock diet. Lower series represents hearing range of 68 guinea pigs fed deficient diet. Length of individual lines indicates range in pitch of audio-signals from 100 to 16,500 cycles per second. Solid portion of line indicates actual range of each animal. Note (1) that no stock animal had seriously diminished hearing range; (2) 19 deficient animals gave no auditory response.



fering from a cold or similar indisposition. Neither were tests made during menstruation. By coincidence none of the subjects were smokers.

Samples of blood were taken after 15 hours' fasting and collected from a fingerprick into a paraffin well. Folin's micro method<sup>3</sup> was used for the glucose determinations, using 1.6% sodium carbonate in place of the sodium cyanide-sodium carbonate solution as recommended by Klendshoj and Hubbard.<sup>4</sup> Readings were made on a Beckman spectrophotometer at 5200 Å. All measurements and read-

ings throughout the study were done by the same analyst. Periodic standard sugar curves were made to check the solutions and the instrument. A search of the literature revealed only 2 observations<sup>5,6</sup> which suggest a relationship of season to concentration of blood sugar in human subjects.

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<sup>3</sup> Folin, O., and Svedberg, D., *J. Biol. Chem.*, 1930, **83**, 85.

<sup>4</sup> Klendshoj, Niels C., and Hubbard, Roger S., *J. Lab. Clin. Med.*, 1939-40, **25**, 1102.

<sup>5</sup> Johnson, Buford, J., *J. Comp. Psychol.*, 1922, **2**, 155.

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<sup>1</sup> Wulzen, R., and Bahrs, A. M., *Physiol. Zoology*, 1936, **9**, 508.

<sup>2</sup> Wulzen, R., and Bahrs, A. M., *Am. J. Physiol.*, 1941, **133**, 500.

<sup>3</sup> van Wagtenonk, W. J., *J. Biol. Chem.*, 1944, **155**, 337.

<sup>4</sup> Simonsen, D. H., and van Wagtenonk, W. J., 1947, **170**, 239.

<sup>5</sup> Folkers, K., *Chem. and Physiol. of Growth*, 1949, 82-86.

ferred with 0.1% sodium acetate,<sup>12</sup> directly into the pars distalis.

In 12 of the 20 experimental animals which survived this operation for 48 hours the copper did not reach the hypophysis; the hypophyseal capsule remained intact. None of these 12 revealed ruptured or hemorrhagic follicles at autopsy. This group included 5 injected with copper acetate in an India ink suspension, and the ink was found in the retropharyngeal region below the sella and in the cavernous sinuses. Of the 8 whose hypophyses actually received copper, 6 ovulated. Two of the 6 had hypophyses penetrated to the top in such a way that some of the copper could possibly have reached the hypothalamus, but in the other 4 no such condition existed. Three of the 8 injected hypophyses were entered with the copper-ink medium and of these, 2 ovulated; but in all 3 most of the ink was located inferior to the sellar region, none above the diaphragma sellae. The effects of mechanical trauma were controlled by a series of 7 animals into each of whose hypophyses a bipolar electrode was inserted,<sup>10</sup> and a second series of 23 rabbits whose hypophyses were injected with acetylcholine- eserine solution.<sup>11</sup> None of the former and only one of the latter ovulated.

At first glance our results, ovulation in

<sup>11</sup> Markee, J. E., Sawyer, C. H., and Hollinshead, W. H., *Endocrinology*, 1946, **38**, 345. 1948, **2**, 117.

<sup>12</sup> Dury, A., and Bradbury, J. T., *Am. J. Physiol.*, 1942, **135**, 587.

75% of 8 rabbits, on intrapituitary injection of copper, might seem unconvincing compared with Harris' 80% of 13 animals on injection of copper into the third ventricle, especially since Harris used a dosage only a third as great as ours. However, most of our injected material backed out of the sella, away from the hypophysis and hypothalamus, while Harris' copper could have reached and stimulated the hypophysis directly rather than the hypothalamus as he assumed. His copper, deposited in the third ventricle, may well have been picked up by the proximal capillary plexus of the hypophyseal portal system and carried by the portal veins directly to the adeno-hypophysis. Brooks' failure to induce ovulation with copper in stalk-sectioned animals is difficult to explain in terms of a direct-action hypothesis, since presumably an adequate arterial blood supply to the adeno-hypophysis remained intact after his operation. The possibility does exist, however, since he could not test them by the mating response and since he supplied no estrogen, that his copper-treated stalk-sectioned animals were anestrus.

To summarize, the intrahypophyseal injection of 1/100th of the systemic dose of copper acetate, an agent whose effect on stimulating the release of hypophyseal LH is not blocked by anti-adrenergic or anti-cholinergic drugs, has led to ovulation in 6 out of 8 rabbits. It is, therefore, concluded that at least part of the copper effect is a direct stimulation of adeno-hypophyseal cells.

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## Reduction of Potassium Tellurite by Living Tissues.\* (17369)

M. WACHSTEIN.

*From the Department of Pathology, St. Catherine's Hospital, Brooklyn, N. Y.*

The property of soluble colorless tetrazolium salts to form insoluble red to purplish formazans as reduction products can be used

for the demonstration and microscopic localization of reducing activity in living tissues.<sup>1-4</sup>

\* This work was done under a grant from the Damon Runyon Memorial Fund for Cancer Research, Inc.

<sup>1</sup> Mattson, A. M., Jensen, C. O., and Dutcher, R. A., *Science*, 1947, **100**, 294.

<sup>2</sup> Pratt, R., and Dufrenoy, J., *Stain Technology*, 1948, **23**, 137.

TABLE I.  
Average Range of Hearing.

	Stock	Deficient
No. of animals	56	64
No. of measurements	176	373
Avg range	10,630	7,328

freedom). It also shows that animals fed stock diet had significantly wider range of response to audio-signals than those fed deficient diet ( $F = 18.34$  with 1 and 118 degrees of freedom).

*Summary.* Guinea pigs fed a diet deficient

in the anti-stiffness factor may ultimately develop inability to respond with the ear-flick to auditory stimuli. At the conclusion of the experiment, 28% of the deficient animals were deaf according to the ear-flick test, in contrast to 0% among the stock animals. It was possible to trace developing deafness according to the ear-flick test in a considerable number of animals maintained on a deficient diet during the experimental period of 7 months.

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### Evidence that Copper Acetate Induces Ovulation in the Rabbit by Direct Stimulation of the Adenohypophysis. (17368)

J. E. MARKEE AND CHARLES H. SAWYER. (Introduced by Philip Handler.)

From the Department of Anatomy, Duke University School of Medicine, Durham, N. C.

Copper salts induce ovulation in the estrous rabbit,<sup>1,2</sup> a species which normally ovulates only after copulation. The natural mating stimulus, which results in the release of luteinizing hormone from the adenohypophysis, involves the hypothalamus and the pituitary stalk<sup>3</sup> and includes both adrenergic and cholinergic components.<sup>4</sup>

Bischoff<sup>5</sup> suggested that copper activated the hypophysis by toxic stimulatory effects on the nervous system. The idea appeared to be confirmed by the results of Brooks,<sup>3</sup> who found that copper failed to induce ovulation in rabbits whose hypophyseal stalks had been severed, and Harris,<sup>6</sup> who reported that very weak dosages of copper acetate injected directly into the third ventricle stimulated the ovulatory response.

We were led to doubt that copper-induced ovulation is purely a nerve-stimulation phe-

nomenon when dibenamine and atropine, in dosages adequate to block the copulation stimulus from reaching the hypophysis,<sup>4,7</sup> failed to prevent copper acetate from activating the release of an ovulatory surge of LH.<sup>8</sup> It therefore seemed desirable to test the hypothesis that copper might exert its stimulatory action directly upon the anterior pituitary.

Sexually mature female rabbits ranging in weight from 2.5 to 4.3 kg were employed in this study. To insure an estrous condition, each animal was treated with 85  $\mu$ g estradiol benzoate on 2 successive days prior to copper administration, for ovulation is not induced by copper in anestrous rabbits.<sup>9</sup> A control series of 10 females revealed that the estrogen alone would not stimulate LH release. The hypophysis was approached parapharyngeally as in earlier studies,<sup>10,11</sup> and 4 attempts were made at 5 or 10 minute intervals to inject a total of 0.15 ml of 0.1% copper acetate, buf-

<sup>1</sup> Ferold, H. L., Hisaw, F. L., and Greep, R., *Am. J. Physiol.*, 1936, **117**, 68.

<sup>2</sup> Emmens, C. W., *J. Endocrin.*, 1940, **2**, 63.

<sup>3</sup> Brooks, C. Mc., *Res. Publ. Assn. Nerv. Ment. Dis.*, 1940, **20**, 525.

<sup>4</sup> Sawyer, C. H., Markee, J. E., and Townsend, B. F., *Endocrinology*, 1949, **44**, 18.

<sup>5</sup> Bischoff, F., *Am. J. Physiol.*, 1938, **121**, 765.

<sup>6</sup> Harris, G. W., *J. Physiol.*, 1941, **100**, 231.

<sup>7</sup> Sawyer, C. H., Markee, J. E., and Everett, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 670.

<sup>8</sup> Sawyer, C. H., and Markee, J. E., unpublished.

<sup>9</sup> Dury, A., and Bradbury, J. T., *Am. J. Physiol.*, 1943, **139**, 135.

<sup>10</sup> Markee, J. E., Sawyer, C. H., and Hollinshead, W. H., *Endocrinology*, 1946, **38**, 345.



FIG. 1.

A frozen section from a rabbit kidney showing tellurium deposition in the proximal convoluted tubules.  $\times 450$ .



FIG. 2.

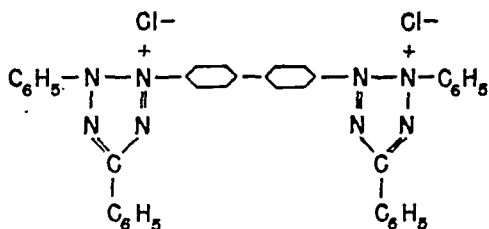
A frozen section from rabbit kidney showing tellurium deposition mainly in ascending limbs of Henle's loops in the outer zone of the medulla.  $\times 150$ .

as human origin. In the cortex, the proximal and distal convoluted tubules, as well as ascending limbs of Henle's loops, showed deposition of tellurium (Fig. 1). Within the outer zone of the medulla, ascending and de-

scending limbs of Henle stained selectively (Fig. 2) while in the innermost portion of the medulla, occasional collecting tubules revealed reducing activity. The intensity of the staining reaction varied considerably from field to

Potassium tellurite introduced into bacteriology by Klett<sup>5</sup> and later utilized by Conradi and Troch<sup>6</sup> for the isolation of diphtheria bacilli, was found to be a suitable reagent for this purpose since reduced insoluble tellurium imparts a distinct black color. A similar reagent had been previously employed by Hasegawa<sup>7</sup> for the determination of the germinability of cereal seeds.

**Method.** A 0.1% solution of potassium tellurite C.P. in M/10 phosphate buffer pH-7.4 gave reproducible and consistent results. For comparison, a saturated solution of "neotetrazolium chloride" obtained from Pannone Chemical Company, Farmington, Conn. in physiological saline solution or phosphate buffer was likewise used. The formula of this compound is:



**Results and discussion.** 24 hour cultures of various bacteria (*E. coli*, *S. typhimurium*, *S. oranienburg*, *S. montivideo*, *S. typhosa*, *Shigella alkalescens*, *Proteus morganii*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *B. subtilis*, *Staphylococcus*, *Streptococcus viridans*, *C. diphtheriae*) grown in 2% peptone water or nutrient broth reduced both "neotetrazolium chloride" and potassium tellurite. 0.2 cc of the reagents were added to 3 cc of the culture medium. A strong reduction was also seen by several strictly anaerobic bacteria, (*C. tetani*, *C. botulinum*, *C. perfringens*) grown in tryptose broth, regardless of whether the cultures after the reagent had been added were

returned to strictly anaerobic condition or left outside in the air. In general, the reduction of potassium tellurite required several hours while that of "neotetrazolium" was apparent within 30-60 minutes. Under the microscope, many of the bacteria as well as the cocci showed similar inclusions with both reagents. The ones following treatment with "neotetrazolium"<sup>1</sup> were larger and coarser, but their general shape and distribution was alike with both preparations.

Suspensions of fresh<sup>8</sup> and of air-dried yeast<sup>9</sup> reduced tetrazolium salts as well as potassium tellurite. Under the microscope, many yeast cells showed fine and coarse granules of similar morphology with both preparations.

Material from various kinds of exudates from human patients were suspended in potassium tellurite and "neotetrazolium chloride" solutions at 37°C. In a considerable amount of polymorphonuclear leucocytes, reducing activity was revealed by the occurrence of purplish red and black granules respectively, of similar appearance and distribution. Thin tissue pieces from rat and rabbit organs as well as from operatively removed human material were suspended in potassium tellurite and "neotetrazolium chloride" solutions and kept at 37°C. Within 15 to 30 minutes, a discoloration took place increasing in intensity within the next hours. The extent of discoloration was similar with both reagents. Kidney tissue, *e.g.*, showed the strongest reaction while intestines reacted only moderately and fat tissue very weakly. A more consistent and deeper penetration of the tissues was however noted with potassium tellurite.

The distribution of reduced tellurium can be studied in frozen sections prepared from formalin fixed tissues cut at 15 to 25  $\mu$  that had been immersed for 8 to 12 hours in the potassium tellurite solution previous to fixation. Paraffin embedding may also be employed but has so far given inferior results. Particularly satisfactory preparations were obtained with kidney tissue of animal as well

<sup>3</sup> Straus, F. H., Cheronis, N. D., and Straus, E., *Science*, 1948, 103, 113.

<sup>4</sup> Antopol, W., Glaubach, S., and Goldman, L., *Public Health Reports*, 1948, 63, 1231.

<sup>5</sup> Klett, A., *Zeitschr. Hyg.*, 1900, 33, 137.

<sup>6</sup> Conradi, H., and Troch, P., *Munch. Med. Wochenschr.*, 1912, 59, 1652.

<sup>7</sup> Hasegawa, K., *Japanese J. of Botany*, 1936, 8, 1.

<sup>8</sup> Kuhn, R., and Jerchel, D., *Ber der Deutschen Chem. Ges.*, 1941, 74B, 949.

<sup>9</sup> Gunz, F. W., *Nature*, 1949, 163, 98.

# Amino Acid Balance at Super-normal Dietary Levels.\* (17370)

H. J. ALMQUIST.

*From The Grange Company, Modesto, Calif.*

It has been pointed out that the same relative proportions of indispensable amino acids to each other must be established to secure the maximal gain possible by the chick on a particular diet, whether the amino acids are supplied as mixtures made up from the single amino acids, as hydrolyzed proteins, or as intact proteins. These findings were based primarily upon protein levels at an approximate optimal value of 20% of the diet.<sup>1</sup> Recently it was shown by Grau that the lysine required to supplement sesame protein for maximal growth of the chick at any protein level from 5 to 30% of the diet was almost directly proportional to the protein level.<sup>2</sup> The present studies were conducted to investigate the relation of dietary protein level to methionine requirement of the chick.

*Experimental.* S. C. White Leghorn, New Hampshire and New Hampshire-Barred Rock Cross cockerel chicks, were fed for one or 2 weeks a practical starting mash, then closely

selected for weight and vigor, divided into groups of 10 to 15 each, and placed on the experimental diets. The soybean meals used were solvent-process meals of known good quality, and with only very slight urease activities. The diets were made up as indicated in Table I.

The chicks were kept for 2 weeks on the experimental diets. Supplements of DL-methionine, and results obtained are indicated in Table II.

Recent analyses of soybean meals for methionine, and, in particular, one thorough study,<sup>3</sup> have shown that modern soybean meals cannot be expected to provide much more than approximately 1.5% methionine, as calculated on the crude protein (% N  $\times$  6.25) basis. More than 0.10% but not more than 0.20% added methionine was needed to permit maximal gain at the 20% protein level. The addition of 0.20% methionine brought the total up to 0.50%, which is the previously estimated minimum requirement standard for chicks.<sup>1</sup> This requirement had been estimated by the use of S. C. White Leghorn chicks. The present data show that this estimate holds equally well for New Hampshire and New Hampshire-Barred Rock Cross chicks. On the other hand, McGinnis and Evans<sup>4</sup> have suggested that New Hampshire chicks may require less methionine than White Leghorns.

The 30% protein diets were not completely supplemented by an addition of 0.20% methionine. It is probable that very nearly 0.30% added methionine, (0.75% total methionine) but not more, was required to permit maximal rates of gain with the 30% protein diets. Evidently, the amount of total methionine needed to support the maximal rate of gain was proportional to the total protein level.

TABLE I.  
Composition of Basal Diets.\*

	I	II
Soybean meal	40S g	617 g
Bonemeal	50 g	50 g
Limestone	10 g	10 g
Paper pulp	20 g	0 g
Salt	10 g	10 g
Animal protein factor conc.	11 g	11 g
Cystine	1.1 g	1.1 g
Riboflavin	4.4 mg	4.4 mg
Ca. pantothenate	11.0 mg	11.0 mg
Niacin	44.0 mg	44.0 mg
Thiamin	4.4 mg	4.4 mg
Choline chloride	660 mg	660 mg
Vit. E	11.0 mg	11.0 mg
Vit. A, U.S.P. units	11,000	11,000
Vit. D, A.O.A.C. units	1,100	1,100
Glucose to	1 kg	1 kg

\* Vitamin quantities indicated are in addition to those present in the soybean meal.

† Supplied by Lederle Laboratories through the kindness of Dr. T. H. Jukes.

\* Presented at the Informal Poultry Nutrition Conference, Detroit, April, 1949.

<sup>1</sup> Almquist, H. J., *J. Nutrition*, 1947, **34**, 543.

<sup>2</sup> Grau, C. R., *J. Nutrition*, 1948, **30**, 99.

<sup>3</sup> Kuiken, K. A., and Lyman, C. M., *J. Biol. Chem.*, 1949, **177**, 29.

<sup>4</sup> McGinnis, J., and Evans, R. J., *J. Nutrition*, 1947, **34**, 725.

field. In general, however, proximal convoluted tubules and ascending limbs of Henle's loop reacted strongest.

Intravital reduction of tellurium in the kidney could also be demonstrated with potassium tellurite, although the reaction was less extensive. Two groups of 6 adult rats received a daily intraperitoneal injection of 0.8 cc of "neotetrazolium chloride" and potassium tellurite solution, respectively, for 16 days. On gross examination, the cortical portion of the kidney was stained. Microscopically, reduction took place only in the proximal convoluted tubules. The depositions of tellurium as well as of the formazan were considerably less intense than in supravitaly stained kidney tissue. With potassium tellurite there was however very little reduction in the liver, while considerable dye deposition took place in the parenchymal cells with "neotetrazolium chloride".<sup>4</sup> The deposition of tellurium in the kidney took place in such a manner as to suggest a localization within large droplets and rods closely simulating the microscopic appearance of kidney sections that had been specifically stained for mitochondria. On chemical examination, Schneider found various enzymes including succinic dehydrogenase to be associated with the mitochondria or large granule fraction from rat liver and kidney homogenates.<sup>10</sup>

The conditions under which potassium tellurite is reduced are closely similar to those under which "neotetrazolium chloride" is acted upon. Boiling, alcohol, acetone, and formalin fixation destroy the reducing ability of animal tissue for both reagents. Antopol and coworkers<sup>4</sup> have noted that in the test tube, cysteine and glutathione at pH-7.0 re-

duce "neotetrazolium chloride" in contrast to cysteine and methionine, thus indicating the importance of active SH groups. An identical behavior was found with potassium tellurite. Correspondingly, treatment with sulfhydryl reagents prevented the reduction of tetrazolium salts by fresh tissue. Similarly, pieces of fresh kidney immersed for one hour in a 0.1% solution of iodocetamide<sup>11</sup> and then incubated up to 20 hours in potassium tellurite or "neotetrazolium" remained completely unstained. Control tissue immersed first in physiological saline solution and then treated with the reagents showed a very strong reaction. There is however considerable lack of specificity for inhibitors of the reduction of tetrazolium salts by living tissues.<sup>12</sup> This would indicate that a number of reducing enzymes, acting possibly on various materials within the cell could be involved in the reducing process.

*Summary.* Reducing activity in living tissue can be demonstrated with the aid of potassium tellurite. Under the microscope, the sites of reduction are indicated by insoluble dark tellurium. Various bacteria, including strictly anaerobic ones as well as yeast and leucocytes from human material, reduced potassium tellurite. Among different mammalian tissue examined, kidney gave the most constant result, permitting histochemical localization in certain portions of the nephron. Active SH groups apparently are essential for the reduction of potassium tellurite by living tissues.

The writer appreciates the technical assistance of Miss Esther Levenkron.

<sup>11</sup> Barron, E. S. G., and Singer, T. S., *J. Biol. Chem.*, 1945, **157**, 221

<sup>12</sup> Fred, R. B., and Knight, S. G., *Science*, 1949, **100**, 169.

<sup>10</sup> Schneider, W. C., *J. Biol. Chem.*, 1946, **165**, 585.

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cial photocell unit. We have adapted the principles outlined by these workers to the use of the compensated circuit Millikan oximeter. The earpiece of this apparatus is clamped over a plastic tube cuvette. Standardization of the red and infra-red-red bias circuits is obtained by galvanometer settings with the neutral filter and with the cuvette filled with oxygen saturated blood respectively.

*Apparatus:* To utilize the photosensitive area of the Millikan earpiece to the maximum, a length of translucent polythene plastic tubing is looped to pass twice through the earpiece perpendicular to the long axis of the photocell limb. The tubing is compressed slightly by thin bakelite plates. Each plate contains an ovoid window in which a microscope cover-glass has been cemented recessed flush with the inside surface of the plate. The diameter of the window perpendicular to the axis of the tubing is determined by the lateral edges of the lumens of the two segments of the polythene loop lying in close proximity. Such dimensions shield light which would pass laterally to the fluid columns. The ends of the ovoid window are arcs of a circle equal

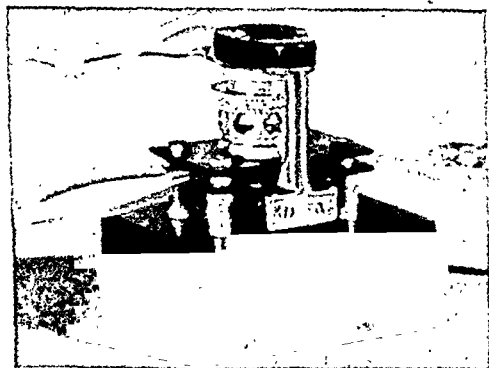


FIG. 1A.

Assembled Cuvette-Earpiece unit. The set screws controlling cuvette thickness fit into the bakelite base and are not seen.

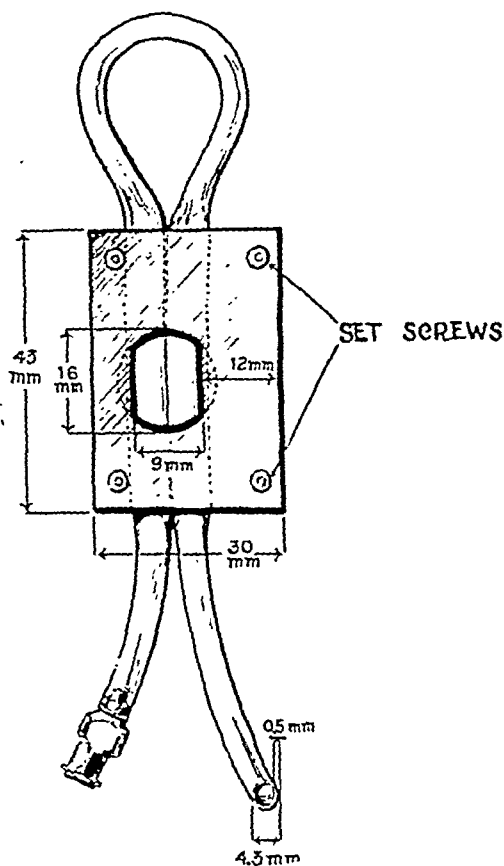


FIG. 1B.

Detail of cuvette light port.

in diameter to the photocell port in the Millikan earpiece. The loop of tubing is compressed to produce a light path distance 1.2 mm through the lumen by a set screw with lock nut at each corner of the bakelite plates. The ovoid window is placed asymmetrically in the plates so that the earpiece is centered over the window when its upright is against one edge of the cuvette plates and the photocell limb is against two of the set screws. This insures a relatively constant location of the earpiece prior to standardization. The plastic tubing is 4.3 mm in external diameter with walls 0.5 mm thick. The tubing, obtained from an ordinary disposable intravenous infusion set (Baxter), may vary slightly in diameter and translucency without affecting the accuracy of the apparatus. When this tubing is compressed to internal diameters

\* Aided by grants from the American Heart Association and the Oregon Heart Association. The oximeter was made available by a generous gift from Mr. Henry Chaney to the Doernbecher Memorial Hospital for Children.

<sup>1</sup> Groom, D., Wood, E. H., Burchell, H. B., and Parker, R. L., *Proc. Mayo Clinic Staff Meetings*, 1948, 23, 601.



TABLE II.  
Relation of Protein and Methionine Levels to the Rate of Gain in Chicks.

Protein in diet, %	Methionine added to diet, %	Total methionine in diet, %	Daily rate of gain		
			S.C. White Leghorn, %	New Hampshire, %	New Hampshire-Barred Rock cross, %
20	0	.30	6.3*	7.1*	7.2
20	.10	.40	6.7	7.6*	7.7
20	.20	.50	7.0*	8.0*	7.9
20	.30	.60	7.0	7.9*	7.9
30	0	.45	6.2*	8.2*	—
30	.10	.55	—	8.4*	—
30	.20	.65	6.7	8.5*	8.2
30	.30	.75	7.2*	8.6*	8.5
30	.40	.85	7.2	8.6*	8.4

\* Average growth rate of duplicate pens.

These results are analogous to those with sesame meal and lysine.<sup>2</sup>

The results discussed above seem sufficient to justify an extension of the concept previously stated by the writer,<sup>1</sup> that the proportions of indispensable amino acids to each other remain relatively the same for maximal efficiency of utilization under varying conditions of level and source of the amino acids. This concept now includes levels of protein intake considerably above what is usually considered the normal requirement for maximal rate of gain.

Since maximal protein efficiency, as indicated by growth rate, is closely related to proportions of amino acids in the diet, even at super-normal protein levels, it would appear, as previously suggested,<sup>1</sup> that "the proportions of amino acids reaching the synthetic regions in the animal are determined largely by the proportions in the diet." The balance of indispensable amino acids in the nitro-

genous increments reaching these cells is more important than the gross protein intake.

Amino acid imbalance in chick diets is not fully correctable by feeding more of the same imbalanced protein. This fact has a bearing on chick assay diets for "animal protein factor," in which approximately 70% soybean meal is used. Even with best qualities of soybean meals, these diets are methionine deficient, and when a material is added which contains both "animal protein factor" and an effective quantity of methionine, as in the case of fish meals, the growth response observed is a resultant of a dual supplementation.

*Summary.* The methionine requirement of the chick for maximal rate of gain on a 30% protein diet is approximately 0.75% of the diet. This value is in direct proportion of the protein levels to the established methionine requirement of 0.50% in a 20% protein diet.

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### Rapid Measurement of the Oxygen Saturation of Whole Blood Samples with the Millikan Oximeter.\* (17371)

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(With the technical assistance of Katherine S. Gullixson.)

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During cardiac catheterization it is frequently desirable to obtain a rapid estimation of blood oxygen saturation in order to orient abnormal locations of the catheter and to de-

termine the uniformity of multiple samples. With this purpose in mind, Groom, Wood, Burchell and Parker<sup>1</sup> have developed a whole blood cuvette oximeter which requires a spec-

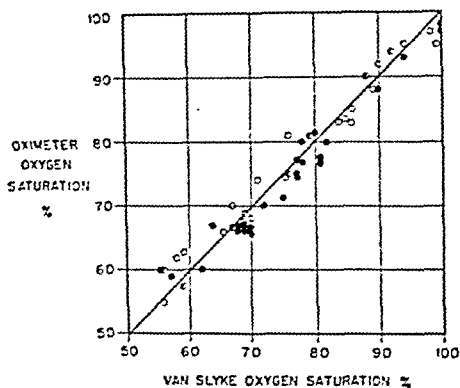


FIG. 3.

Comparison of oxygen saturation determinations on 54 blood samples, as determined by the oximeter and by the Van Slyke manometric method. Black points represent polythemic blood samples having an oxygen capacity from 29.1 to 33.2 volumes %. Half-black circles represent anemic blood samples having an oxygen capacity of 9.0 volumes %. Circles represent blood samples of intermediate hemoglobin concentration (14.0 volumes % to 23.0 volumes % oxygen capacity).

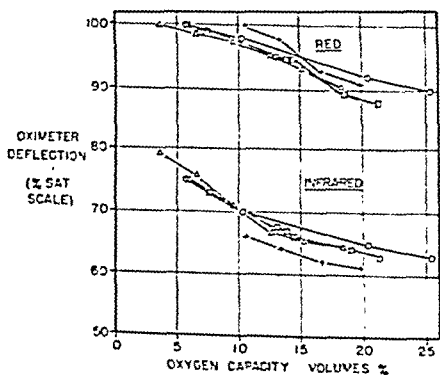


FIG. 4.

Variations of apparent oxygen saturation produced by change of hemoglobin concentration after standardization of the oximeter circuits. Fully saturated blood was diluted serially with plasma. The most dilute blood sample was used to set the bias circuit (RED) at 100% saturation, and, without change of galvanometer settings, the fully saturated samples of higher oxygen capacity measured. Although the true oxygen saturation of all samples was 100%, there was a fall in oxygen saturation as measured by the oximeter with more concentrated blood specimens, indicating incomplete compensation for changes of hemoglobin concentration.

minimizes exhaustion of the barrier layer photocells.

**Results.** Comparison of blood oxygen saturation determinations by the oximeter and

by the manometric method of Van Slyke and Neill<sup>3</sup> is shown in Fig. 3. The standard error between observations by the two methods on 54 blood samples was 2.4% oxygen saturation. Determinations were made using human blood samples taken at the time of right heart catheterization and samples of dog blood desaturated by tonometric equilibration with nitrogen and air. The oxygen capacity of these samples varied from 9.0 volumes % to 33.2 volumes % without affecting accuracy significantly. The effect of wide variations of hemoglobin concentration following standardization is shown in Fig. 4. Blood from stock dogs was serially diluted with dog plasma and equilibrated with air at room temperature. The most dilute blood-plasma mixture was used to set the bias circuit at 100% saturation. The apparent oxygen saturation reading and infrared galvanometer reading were then recorded for the more concentrated fully saturated blood-plasma mixtures. The observations during four such experiments are plotted against oxygen capacity in Fig. 4. A change of oxygen capacity on the order of 10 to 20 volumes % is required to produce a change of 10% in the observed oxygen saturation. Thus although the bias circuit does not completely compensate for wide fluctuations in hemoglobin concentration, any slight variation of hemoglobin concentration due to fluid infusion and serial sampling during catheterization should not affect saturation readings appreciably.

If it can be shown that the light transmission of the empty cuvette remains constant, modification of this recording circuit would permit measurement of the entire length of galvanometer deflection on introduction of blood into the cuvette and consequently would allow direct reading of oxygen saturation without the step of standardization with oxygen-saturated blood. However, the assumption of constancy of light transmission is not warranted since after several hours' use there is gradual fogging of the plastic tube exposed to the 6 v earpiece lamp. After several hours' use, the plastic tubing is replaced.

<sup>3</sup> Van Slyke, D. D., and Neill, J. M., *J. Biol. Chem.*, 1924, **61**, 523.

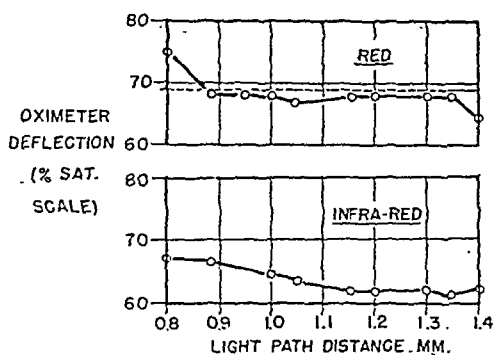


FIG. 2.

Light transmission properties with varying light path distance. At each light path distance the red infra-red bias circuit was standardized with fully saturated blood in the cuvette. Aliquots of a nitrogen desaturated sample of the same blood then produced galvanometer deflection. The lower portion of the diagram shows the galvanometer deflection produced by activation of the infra-red photocell alone. The upper portion of the diagram (RED) shows galvanometer deflection produced by the bias circuit measuring oxygen saturation. The dotted line indicates oxygen saturation of the nitrogenated blood sample as measured by Van Slyke manometer. Between light path distances of 0.88 mm and 1.35 mm a constant half scale galvanometer deflection is produced with desaturated blood having an oxygen capacity of 23.1 volumes %.

shown in Fig. 2, an area of absorbing solution on the order of 120 sq mm is exposed to light. One end of the tubing is fitted with the hub of an 18 gauge hypodermic needle, which has been drilled to larger bore. The set-screws of the cuvette fit into a heavy bakelite base.

A commercial oximeter serves as the recording device and as power unit for the earpiece lamp.<sup>1</sup> The earpiece utilizes a light filter in the red range and infra-red range rather than the red and green light range of the original Millikan earpiece. The bias circuit of this apparatus results in direct recording of oxygen saturation; such a circuit is described in Millikan's original communication<sup>2</sup> as a single scale oximeter with automatic adjustment for ear thickness. Currents are recorded with a mirror galvanometer (Rubicon 3415). The scale, 10 cm in length, is calibrated directly in percent oxygen saturation from 50% to 100% in 5% scale divisions.

ation from 50% to 100% in 5% scale divisions.

**Procedure.** A warm-up period of 5 to 10 minutes is required for the earpiece circuits. A standard galvanometer deflection of the infrared cell circuit ("green" circuit) alone is set using variable resistance  $R_1$  with a standard filter in the light path of the earpiece (Coleman A Filter). The earpiece is clamped firmly in place over the cuvette. A blood sample is drawn by catheter from the patient and saturated with tank oxygen at room temperature. With this saturated blood in the cuvette, the red-infra-red bias circuit is standardized at 100% saturation with variable resistance  $R_2$ . The oximeter remains stable over a period of hours, but bias circuit standardization may be repeated at intervals. Once standardization is completed, optical arrangements should not be altered by change of position of setscrews, earpiece screw or earpiece. The oximeter will faithfully reproduce saturation values over a wide range of thickness of the whole blood column (Fig. 2) but, following any change of light path distance, new standardization of the bias circuit with fully oxygenated blood is required. A portion of the unknown blood sample, collected in an oiled heparin syringe, is saved in a fluoride tube for Van Slyke manometric analysis. The last 2 ml of blood contained in the syringe are passed into the cuvette. This quantity of blood is sufficient to fill the cuvette allowing 0.5 ml to flush the lighted segments of plastic tubing. Both infra-red and bias circuit galvanometer readings are made. The infra-red deflection records changes in total hemoglobin concentration. The bias circuit reading indicates the oxygen saturation. Constancy of the infra-red galvanometer reading for successive blood samples indicates absence of air or oil bubbles in the light path and constancy of hemoglobin concentration. The entire length of the plastic tubing can be inspected for trapped air or oil at any time during the procedure. Usual room lighting produces no significant error in saturation determinations. Between readings the cuvette is left filled with blood being flushed with isotonic sodium chloride solution and air just prior to introduction of the next sample. This maneuver

<sup>1</sup> Coleman Anoxia Photometer Model 17A. Coleman Instrument Company, Maywood, Ill.

<sup>2</sup> Millikan, G. A., *Rev. Sci. Inst.*, 1942, **13**, 434.

TABLE I.  
Plasma and Urine Amino Acid Levels Prior and Subsequent to Injection of 131 ml of a Mixture of 10 Amino Acids (VUJ-N).  
Subject, K.H. ♂ 131 Lbs.

Amino acid inj. (mg L isomer)	Leucine 1640	Lysine 1390	Isoleucine 1073	Valine 753	Histidine 524	Methionine 524	Phenylalanine 341	Threonine 245	Arginine 219	Tryptophan 30
Time (min.)										
0	1.6	3.2	0.8	3.7	2.1	0.2	1.3	1.6	1.3	1.3
15	6.2	7.8	4.5	10.4	3.2	2.0	1.8	2.4	3.5	1.8
30	4.7	5.8	2.4	6.7	2.9	1.2	1.7	2.4	2.0	1.7
60	2.9	4.2	1.5	4.9	2.4	1.0	1.6	1.7	1.2	1.4
120	2.2	3.4	0.9	3.8	2.0	0.4	1.4	1.4	1.2	1.2
180	2.1	3.0	0.9	3.7	1.8	0.3	1.3	1.2	1.2	0.9
2 hr prior to inj.	78	200	24	104	4000	24	64	320	440	310
3 hr post inj.	363	2406	91	182	13600	182	647	1816	1020	363
Diff.	285	2206	67	78	9600	158	583	1496	580	53
% of inj. dose excreted in 3 hr	0.05	0.48	0.02	0.03	5.50	0.09	0.51	1.83	0.79	0.53

nolds, and Baumann.<sup>6</sup> *Leuconostoc mesenteroides*, P-60 was employed as the assay organism for all amino acids except threonine and arginine for which *Streptococcus fecalis* was preferred.

**Results and discussion.** In Table I there are listed the absolute amino acid levels (*L*-isomers, mg per 100 ml plasma) which were observed before and at various intervals after the amino acid mixture was infused in a normal male subject chosen as typical of 5 experiments. This table also lists the quantities of the 10 amino acids ( $\mu$ g per hour) excreted during the 2-hour period preceding the infusion as well as for the 3-hour period during which blood samples were taken. From this data the percentage of the injected dose which was excreted during the period of observation was calculated.

The amino acid pattern infused is also compared (Fig. 1) to that obtaining in the plasma of 2 subjects at various intervals subsequent to the injection. For this purpose each amino acid is first corrected for its fasting level and all concentrations are then expressed as a percentage of the total amino nitrogen measured in the form of the 10 amino acids. In order to compare the excreted with the infused pattern the individual contribution (% amino nitrogen) of each amino acid to the total 3-hour amino nitrogen excretion of the 10 amino acids is also shown in Fig. 1.

It will be noted that while the amino acid levels at 15 minutes after the injection are more or less elevated in proportion to the amount injected, subsequently the levels fall rapidly, in some instances decreasing to substantially lower than the fasting levels. It is possible that this latter effect is referable to a synthetic process which requires some of the original amino acid supply of the body. The comparison of the pattern of 10 amino acids in the plasma at various intervals after the injection with that originally infused reveals some differences between the two subjects in the rate of disappearance of individual acids. In Subject K.H., the preponderance of leucine, valine, and methionine is noteworthy.

<sup>6</sup> Steele, B. F., Sanberlich, H. E., Reynolds, M. S., and Baumann, C. A., *J. Biol. Chem.*, 1949, **177**, 533.

Standardization with oxygenated blood prior to each period of use eliminates this source of error. In addition the introduction of a more sensitive galvanometer would reduce the portability of the apparatus.

Agreement within 4.8% saturation between oximeter and manometric values can be expected ninety-five percent of the time. However, we have not used the oximeter other than as a scouting device at the time of catheterization checking all results with Van Slyke an-

alyses prior to calculation of flow components.

*Summary.* (1) A polythene tubing cuvette is described which adapts the Millikan oximeter for measurement of oxygen saturation of drawn samples of whole blood.

(2) Oxygen saturation values agree with Van Slyke manometric values with a standard error of 2.4%.

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## Intravenous Amino Acid Tolerance Studies in Humans.\* (17372)

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The introduction of specific and sensitive microbiological methods for the assay of amino acids has made possible the further study of many fundamental problems in the metabolism of these nutrients. The increasing clinical use of amino acids in parenteral nutrition, particularly in surgery, further emphasizes the necessity for more detailed studies on the fate of these substances in the human organism. For this purpose the rates of disappearance of intravenously administered amino acids have been investigated. The problem was first studied with *DL*-methionine as a test amino acid.<sup>1-3</sup> In view of the recent work on the importance of the simultaneous presence of all of the essential amino acids if maximal nitrogen retention is to occur,<sup>4</sup> it was felt that further tolerance studies should be conducted with a mixture

of the 10 amino acids originally thought to be indispensable to man. The present paper reports the results of such studies on normal human males in intravenous tolerance to 10 amino acids when injected simultaneously. Included also are the data on the urinary excretion of the injected amino acids.

*Experimental.* The amino acid preparation used was the VUJ-N mixture kindly supplied by Merck & Company. The product contained varying amounts of the 10 indispensable amino acids plus additional glycine. After a 12-hour fast, blood samples were withdrawn for determination of fasting levels of the 10 amino acids. The amino acid mixture was then injected intravenously as rapidly as possible using 50 ml syringes (about 4-5 minutes) at a level of 1 ml per pound body weight. Blood samples were taken at 15, 30, 60, 120 and 180 minutes after the injection, and urine excreted during the 2-hour period immediately prior to the injection as well as during the period when the bloods were taken was also collected for assay. Ten amino acids were then determined by microbiological methods on heat deproteinized plasma from heparinized blood, and on the urines. The basal medium used was either that of Henderson and Snell,<sup>5</sup> or Steele, Sauberlich, Rey-

\* Presented before the Society of University Surgeons, Tenth Annual Meeting, San Francisco, March 24, 1949.

<sup>1</sup> Harper, H. A., Kinsell, L. W., and Barton, H. C., *Science*, 1947, **100**, 309.

<sup>2</sup> Kinsell, L. W., Harper, H. A., Barton, H. C., Michaels, G. D., and Weiss, H. A., *Science*, 1947, **100**, 589.

<sup>3</sup> Kinsell, L. W., Harper, H. A., Barton, H. C., Hutchin, M. E., and Hess, J. R., *J. Clin. Invest.*, 1948, **27**, 677.

<sup>4</sup> Geiger, E., *J. Nutr.*, 1948, **34**, 97.

<sup>5</sup> Henderson, L. M., and Snell, E. D., *J. Biol. Chem.*, 1948, **172**, 15.

exceeded normal without any resulting marked increase in excretion. In the present experiments, the rate of disappearance of leucine from the blood is much greater than that of histidine but these differences are not reflected in the urine. It is therefore felt that studies of amino acid changes in the blood plasma may be more valid for the purpose of an investigation of utilization of various mixtures.

A comparison of tolerance to the same quantities of amino acids when infused at a constant rate over a one-hour period is now in progress. The results will be reported in a later communication.

**Summary.** A mixture of the 10 indispensable amino acids has been injected intravenously in normal human male subjects and the plasma levels of these 10 acids as well as the urinary excretion, prior and at various intervals subsequent to the injection, were determined microbiologically. The pattern of amino acids excreted is shown to bear no relationship to that infused.

The work here reported has been carried out with the active cooperation of Drs. H. J. McCorkle, of the University of California Medical School, and H. L. Silvani, Frank Choy, and their associates at The Veterans' Hospital, Fort Miley.

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### Intravenous Administration of Massive Dosages of Estrogen to the Human Subject; Blood Levels Attained. (17373)

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The employment of estrogens in the management of cases of cancer of the breast<sup>1</sup> and prostate<sup>2</sup> necessitates our further knowledge of the biological effects of the estrogens when administered in varying dosage and by various routes.

Few data are available concerning the effects of intravenous administration of estrogen in the human subject. Loeser<sup>3</sup> injected as much as 10 mg estrone, 25 mg estradiol, or 150 mg stilbestrol intravenously in propylene glycol and observed an elevation of intra-uterine temperature presumably attributable to increased uterine blood-flow. He noted no toxic side reactions and indicated that the medication was well tolerated. Abarbanel<sup>4</sup> reported the administration of small doses of estrone sulfate for the control of uterine bleeding.

We wish to report on the clinical tolerance of intravenous infusions of massive dosages of an aqueous solution of conjugated natural estrogens.\* We have also determined by bioassay the blood levels attained immediately following infusion and at fixed intervals after treatment.

**Materials and methods.** Thirteen female and 9 male subjects have been studied. Their age, race, and clinical diagnosis are indicated in Table I. All patients were at bed rest when treated. Intravenous infusions in the dose and volume indicated were carried out in the customary manner employing the ante-cubital vein. A buffered aqueous solution of conjugated estrogens in the form of a concentrate from pregnant mare's urine was mixed with normal saline so that a final concentration of from 0.5 to 2 mg equivalents of estrone sulfate per cc was obtained for injection. The rate of flow was so adjusted that the total dose was administered in from 35 to 60 minutes. Each patient's temperature, pulse,

<sup>1</sup> Nathanson, I. T., *Surg. Clin. N. A.*, 1947, 27, 1144.

<sup>2</sup> Huggins, C., Stevens, R. E., and Hodges, C. V., *Arch. Surg.*, 1941, 43, 209.

<sup>3</sup> Loeser, A. S., *J. Obst. and Gynec. Brit. Emp.*, 1948, 55, 17.

<sup>4</sup> Abarbanel, A. R., paper No. 71; read by title; Assn. for Study of Internal Secretions, June, 1949.

\* The preparation used was "Premarin" (injectable) kindly supplied by the Ayerst, McKenna & Harrison Ltd., through the courtesy of Drs. G. H. C. McKeown and R. C. Reifstein, Jr.

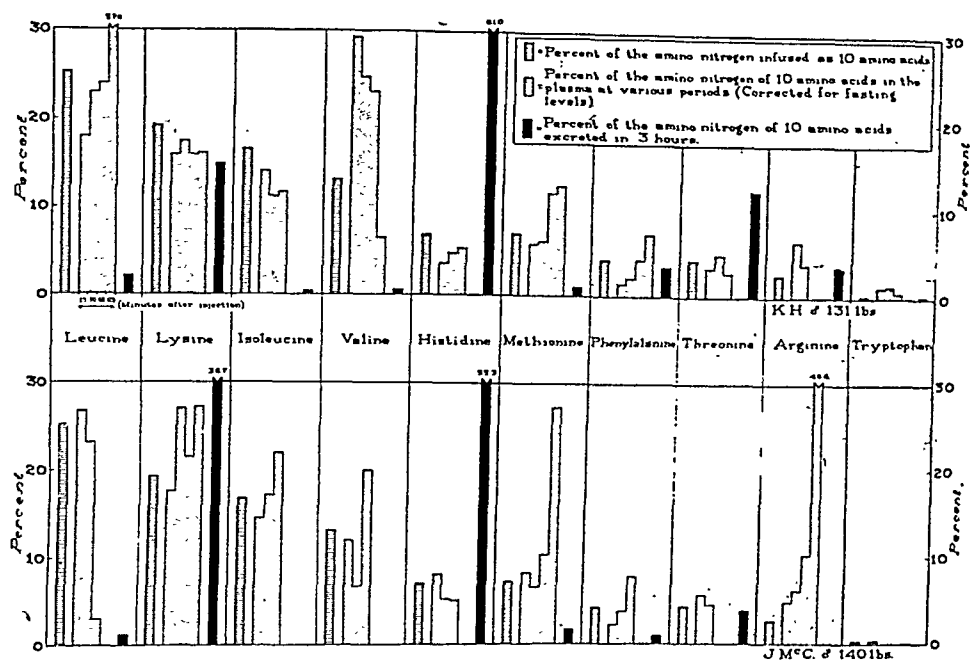


FIG. 1.

Comparison of amino acid pattern infused with that of the plasma at intervals subsequent to injection. The individual contribution of each amino acid to the total 3-hour amino nitrogen excretion of 10 amino acids is also shown. All concentrations are expressed as a percentage of the total amino nitrogen measured in the form of 10 amino acids.

In Subject J. McC., lysine, methionine, and arginine are responsible for the excess over the infusion mixture, in the later periods. Only the persistence of methionine is common to both subjects. The other acids retain a close resemblance to the infusion mixture during most of the period of observation. The effect of a change in the concentration of any one amino acid on the uptake of the others would be of interest. This experiment is planned as an extension of the basic tolerance studies.

The pattern of amino acids excreted bears no relationship to that infused. Very little quantitative data on the renal threshold for amino acid excretion in humans is available. One fact that is well established, however, is that the reabsorptive capacity of the kidney for the natural *L* isomers, in contrast to the *D* isomers, is relatively high. At the infusion levels used in these experiments, histidine, lysine and threonine accounted for the majority of the urinary amino nitrogen measured. One could therefore disregard the losses by the renal route of the other amino acids.

Silber and his collaborators<sup>7</sup> have infused into dogs an amino acid mixture similar to that used in our experiments and studied the pattern of the essential amino acids excreted in the urine. They also point out that the urine pattern did not resemble the pattern infused nor was it greatly altered by protein depletion. However, in a subsequent paper, Silber<sup>8</sup> by infusing 3 different amino acid mixtures in dogs was able to show a relationship between intake and excretion and suggested that this approach might be useful in designing amino acid mixtures for specific types of patients. From our experiments it would seem that because of the high renal threshold for most amino acids, the urine fails adequately to reflect either blood levels or extent of uptake by the tissues. In liver disease we have repeatedly observed plasma levels of *L*-methionine, for example, which far

<sup>7</sup> Silber, R. H., Seeler, A. O., and Howe, E. E., *J. Biol. Chem.*, 1946, **164**, 639.

<sup>8</sup> Silber, R. H., Howe, E. E., and Porter, C. C., *Trans. N.Y. Acad. Sci.*, 1948, Ser. II, **10**, 277.

C.M.	W.M.	63	4/15	200*	.0	28.4	7.6	3.2	.0	.0	Cancer of prostate with metastases
			22	200*	.0	26.4	8.4	2.4	.0	0	
			24	200*	.0	23.8	6.8			0	
			26	200*	.0	21.0		2.8		0	
			28	200*	.0	24.4		6.4	2.4	0	
			5/ 3	200*	.0	26.4	4.0		3.8	0	
			5	250*	.0	25.6		6.8	2.8	0	
											Massive, fungating breast cancer; marked debility
M.W.	C.F.	58	5/10	200*	.0	22.4	6.8	3.6	.0	0	
			17	200*	.0	21.2	7.6	4.8	5.0	0	
			25	300*	.0	33.0	14.0	8.4	7.2	0	
									4.0	0	
L.J.	C.F.	79	5/25	200*	.0	23.8	6.0	.0	.0	0	Advanced breast cancer; marked senile degeneration
			27	300*	.0	38.0	12.8		2.0	0	
			31	400*	.0	38	14.4	7.6	3.4	0	Breast cancer with osseous metastases
A.F.	W.F.	45	6/ 2	300*	.0	31	.0	.0	.0	0	
			9	400*	.0	32	13.2	3.2	.0	0	
											Breast cancer with osseous metastases
M.M.	C.F.	71	6/ 2	300*	.0	35	13.6	6.8	3.4	0	
			9	300*	.0	45	14.8			(a)	
J.F.	G.M.	66	6/ 9	175§	.0	35	3.6			0	Cancer of prostate
G.P.	W.F.	73	6/10	250*	.0	35	10.0	4.2	.0	0	Severe rheumatoid arthritis
			14	300*	.0	39.8	14.4	7.6	2.8	0	
			15	300*	.0	33				0	
			16	300*	.0	32				0	
			17	300*	.0	37				0	
F.J.	O.M.	44	6/10	250*	.0	38	14.0	6.0	2.0	0	Severe rheumatoid arthritis
			14	300*	.0	36	12.8	5.6	2.0	0	
			15	300*	.0	39.8				0	
			16	300*	.0	34				0	
			17	300*	.0	35				0	
											Severe rheumatoid arthritis
J.D.	C.F.	43	6/10	250*	.0	39	12.8	3.2		0	
			14	300*	.0	32	13.2	6.6		(b)	
			15	300*	.0	48				0	
			16	300*	.0	35				0	
			17	300*	.0	35				0	
D.M.	C.F.	59	6/16	207	.0	19			.0	0	Advanced cervical cancer; frozen pelvis
F.F.F.	W.F.	68	6/15	250*	.0	45	14.8	8.0	6.0	0	
			16	225*	.0	38			7.8	0	
			17	300*	5.8	43		18.0	7.8	0	

• Given in 200 cc normal saline.

(b) Nausea for 6-8 hours after infusion.

(c) Died following infusion—seo text.

(c) Pled removing intrusion—see text. Values less than 1  $\mu\text{g}$  doubtful and indicated by 0.0.



TABLE I. Intravenous Estrogen Administration.

Patient	Sex and color	Age	Date treated	Total dose (mg)	Serum level of estrogens ( $\mu$ g)							Reaction	Remarks
					Pre.	Post	2 hr	4 hr	6 hr	8 hr	24 hr		
E.T.	C.F.	62	2/16/49	200*	.0	36	8.0	3.2				0	Cancer of breast, with wide local extension; arteriosclerotic degeneration of the brain
				200*	.0	24.4	12.0	3.2				0	
				200*	.0	38				.0		0	
				200*	.0	30	5.6	.0	.0	.0	.0	0	
				15 300*	.0	35	7.6	4.0	.0	.0	.0	0	
				18 300*	.0	39	4.3	2.4	.0	.0	.0	0	
				5/17 100†	.0	23.2				.0	.0	0	
				25 300*	.0	37		5.6		.0	.0	0	
				27 400*	.0	35	18.4	8.6	6	3.8	.0	0	
				31 400*	.0	38	16.0	7.0		.0		0	
N.H.	C.F.	76	2/16	200*	.0	21.8						0	Cancer of breast, pulmonary and osseous metastases; hypertensive heart disease
				200*	.0	22.4						0	
				400*								(c)	
R.A.	W.F.	53	3/ 4	200*	.0	28.4	5.6	5.4	2.4	.0		0	Bilateral, inoperable breast cancer with metastases
				200*	.0	19.0			2.2	.0	.0	0	
				15 300*	.0	23.8		3.6		.0	.0	0	
				18 300*	.0	39	5.6	3.2	3.2	.0	.0	0	
J.F.	C.M.	55	3/17	200*	.0	24.4	7.2				.0	0	Carcinoma of prostate; osseous metastases; moribund
				200*	.0	25.8	6.0			7.4	.0	0	
J.S.	W.M.	67	3/17	200*	.0	23.8	7.0					0	Carcinoma of prostate
				200*	.0	22.4	3.6	.0	.0	.0		0	
				240*	.0	21.2	2.0	.0	.0	.0	.0	0	
				31 200*	.0	17.6		.0	.0	.0	.0	0	
				5/ 5 200*	.0	25.6		2.4		.0	.0	0	
J.W.	W.F.	41	3/28	250*	.0	21.2		2.0		.0	.0	0	Cancer of breast; pulmonary metastases with pleural effusion
				100†	.0	22.4	.0	.0	.0	.0	.0	0	
				132†	.0	22.0	3.2		.0	.0	.0	0	
C.D.	W.M.	65	4/ 4	200*	.0	24.0	10.0		7.6			0	Cancer of prostate, huge abdominal masses
												0	
L.S.	W.M.	59	4/ 4	200*	.0	21.6	6.0		.0			0	Cancer of prostate
												0	
A.T.	W.M.	68	4/ 8	200*	3.2	25.6	9.6	.0		.0		0	Carcinoma of prostate with metastases to bone and lymph node
												0	
P.C.	C.M.	81	4/11	200*	.0	23.6	8.1	4.2		.0		0	Carcinoma of prostate

fusions were well tolerated. Serum levels of estrogen as determined by bioassay varying from 17.6 to 48  $\mu\text{g}$  were obtained and only negligible quantities of estrogen remained in the blood after 8 hours.

The feasibility of rapidly inducing a high

serum estrogen level by intravenous infusion is demonstrated. The clinical usefulness of this form of estrogen therapy in carcinoma of the breast and prostate remains to be evaluated.

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## Blood Pressure in the Rat.\* (17374)

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The work of Kersten *et al.* on the indirect measurement of blood pressure in the rat<sup>1,2</sup> will increase the use of rats for studying various phases of the hypertension problem. If rats of different ages are to be used it should be remembered that "a tendency to a slight rise in blood pressure with age" has been noted.<sup>3</sup> It was the scope of the following investigations to establish accurate data which the literature does not provide.

Rats of the Long-Evans strain were kept on Friskies and water. The blood pressure was measured in unanesthetized, unheated animals with the foot method described by Kersten, Brosene, Ablondi and SubbaRow.<sup>1</sup> Readings obtained about 1 minute apart in the same animal rarely differed more than  $\pm 3$  mm Hg.

Fig. 1 shows that the systolic blood pres-

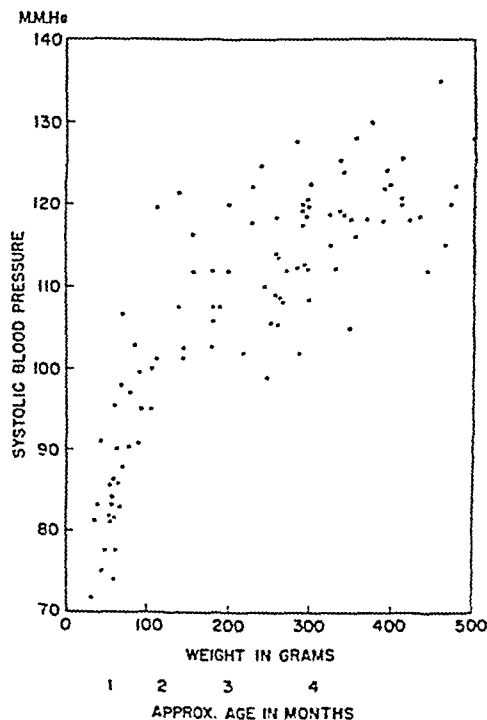


FIG. 1.

Systolic blood pressure in rats of different weights and ages.

sure increases with age in the growing rat. Within the first 2 months up to a weight of 150 g the pressure increases rapidly. A slower, but still progressive rise is noted in rats weighing 200 to 350 g.

The age conditioned differences in the sys-

\* Aided by a grant from the Life Insurance Medical Research Fund.

<sup>1</sup> Kersten, H., Brosene, W. G., Jr., Ablondi, F., and SubbaRow, Y., *J. Lab. and Clin. Med.*, 1947, **32**, 1090.

<sup>2</sup> Ablondi, F., SubbaRow, Y., Lipchuck, L., and Personens, G., *J. Lab. and Clin. Med.*, 1947, **32**, 1099.

<sup>3</sup> Griffin, John A., and Parris, E. J., *The Rat in Laboratory Investigation*, J. B. Lippincott Co., Philadelphia-Montreal-London, 1942, p. 286.

<sup>4</sup> An apparatus was obtained from the Lederle Laboratories Division, American Cyanamid Company, through the courtesy of the late Dr. Y. SubbaRow.

respiration and blood pressure were recorded before, during and after the infusion. Blood samples were drawn for estrogen bioassay just prior to the beginning of the infusion, at the conclusion of the injection, and at stated intervals throughout the subsequent 8 to 24 hours. The drawn blood was permitted to clot and the serum promptly prepared.

The estrogen content of the sera was determined by a modification of the uterine weight method of Lauson *et al.*<sup>5</sup> Twenty-one day-old female rats of the N.I.H. or Holtzman strain were ovariectomized and were given the proper dilution of serum in 0.25 cc twice daily for 2 days. Twenty hours after the last injection the test animals and uninjected controls were autopsied, the uterus dissected out, freed of fluid and weighed to the nearest milligram on a Roller-Smith torsion balance. The estrogen content of the patients' sera was calculated on the basis of the activity of an aqueous dilution of the same preparation of conjugated estrogens that had been employed for the clinical studies. Duplicate and multiple determinations at various levels have indicated an approximate error of 10% in our estrogen bioassays.

*Results and discussion.* The clinical reaction of 67 infusions of from 100 to 400 mg each was observed. All but 3 infusions were attended by no subjective reactions on the part of the patient. One patient (J.D.) who had received 200 mg complained of nausea for 6 hours following the injection. Another patient (M.M.) who had received 350 mg suffered a mild chill necessitating the discontinuation of the infusion. The third patient (N.H.) who was in a badly debilitated state from advanced metastatic breast cancer and hypertensive heart disease died shortly after the administration of 400 mg of conjugated estrogens. The terminal clinical picture simulated that of cerebrovascular accident. Careful autopsy examination, including study of the brain, revealed no apparent basis for this patient's death. In view of her general debility and the total lack of reaction on the part of other patients to identical treatment, it may be considered that her death was not

causally related to the estrogen administration.

The pulse, temperature, blood pressure and respiration in the remaining cases were not significantly affected. The apprehension attendant upon the venipuncture induced a slight but transient rise of blood pressure in some cases, particularly upon the first infusion. This momentary effect subsided rapidly and in most cases was not observed during subsequent infusions. Hyperpyrexia was observed only in the one patient (M.M.) who had experienced a reactive chill.

On the whole, the infusions were remarkably well tolerated in spite of the very high doses employed. Moreover, it should be noted that most of our subjects were all bedridden, debilitated individuals with advanced cancer of the breast or prostate and even greater tolerance might be anticipated from patients in better general clinical condition.

The blood levels of estrogen attained immediately following the infusion varied from 17.6 to 48  $\mu\text{g}$  per cc and rapidly dropped off to negligible levels in most cases after 8 hours. This indicates a fairly ready dilution of the estrogen in the blood and a rapid removal therefrom. Studies on the urinary excretion and metabolic fate of the injected estrogen will be reported subsequently.

These preliminary observations indicate the feasibility of procuring an inordinately high blood level of estrogen in a short time. The quantitative data will permit an estimate of the rate of estrogen infusion which may be required for the maintenance of such levels for longer periods of time. The potential usefulness of such intensive estrogenization in patients with prostatic and breast cancer remains to be determined. We have noted no material clinical effect on the malignant process from the limited number of infusions thus far administered. The evaluation of such potential effects will require more prolonged periods of sustained treatment and observation.

*Summary.* Thirteen female and 9 male subjects have been given a total of 67 intravenous infusions of from 100 to 400 mg of conjugated natural estrogens dissolved in 200 cc of normal saline. In the main, these in-

<sup>5</sup> Lauson, H. D., Heller, C. G., Golden, J. B., Severinghaus, E. L., *Endocrinology*, 1939, 24, 35.

TABLE I.  
Ten Normal Marrows Studied by the Dry Technic.

	Fields with megakaryocytes		Fields without megakaryocytes		% of meg. with thrombocytes
	Thrombocytes per 100 megakaryocytes	Clumps of thrombocytes per 100 meg.	Thrombocytes per 100 fields	Clumps of thrombocytes per 100 fields	
Areas with structure	387	16	369	8	25
Areas with no structure	1496	24	887	8	65

TABLE II.  
Four Cases of Idiopathic Thrombocytopenic Purpura Studied by the Dry Technic.

	Fields with megakaryocytes		Fields without megakaryocytes		% of meg. with thrombocytes
	Thrombocytes per 100 megakaryocytes	Clumps of thrombocytes per 100 meg.	Thrombocytes per 100 fields	Clumps of thrombocytes per 100 fields	
Areas with structure	nil	Prespleneectomy nil	nil	nil	nil
Areas with no structure	9	nil	7	nil	nil
Areas with structure	36	Postspleneectomy nil	13	nil	2
Areas with no structure	1117	12	475	nil	59

ond syringe that had been washed out with sodium citrate was used to aspirate a second sample of marrow, the further steps being those just described.

*Dry method.* The puncture was performed as before. A perfectly dry needle was used and a dry syringe attached to it. A sample of marrow, usually 1 cc or less, was rapidly aspirated. A small amount was then quickly expressed through another needle on to a clean cover slip. Excess fluid was withdrawn by means of the syringe, and the film was spread by means of a second cover slip. The films were stained with Wright's stain, and mounted on glass slides.

Ten normal marrow films made by the dry technic were first studied. Regardless of the technic used, it is common to find areas in which there is some preservation of marrow structure; fat spaces may be seen in many instances, although at other times there is merely a large aggregation of cells which can be observed with the naked eye. There are also other areas in which the cells lie quite free as in a blood film. The percentage of

megakaryocytes that showed budding in areas with marrow structure and in areas without such structure were estimated separately. In addition, each time a megakaryocyte was encountered, another field 3 oil immersion fields distant from it was studied, and the number of thrombocytes contained therein was enumerated. Sometimes it was possible to count individual thrombocytes. At other times they had to be counted as clumps. In the normal marrows, a total of 100 megakaryocytes was examined in areas with marrow structure, and 100 in areas without such structure. In the other films it was possible to examine 25 megakaryocytes in each such area, and in some marrows, where budding was scanty, larger numbers of cells were used. Budding was recorded as being present when it appeared to be fairly definite, but this is a matter about which there may be divergence of opinion between different observers. The mere presence of megakaryocytes and thrombocytes in the same field was not considered to indicate true budding. The findings obtained by the dry technic in ten normal marrow speci-

tolic blood pressure in the rat are of a magnitude which deserves attention. A pressure of 120 mm Hg, normal for a rat weighing 300 to 500 g, is definitely hypertensive for a rat weighing less than 100 g.

The blood pressure in man increases in the corresponding age groups of infancy and early childhood in a similar fashion.<sup>4,5</sup> The consid-

eration of this fact is common practice in pediatric experience.

*Summary.* The systolic blood pressure has been recorded in 100 unanesthetized, unheated rats of various ages. It increases with weight and age and normal values were established.

<sup>4</sup> Graham, Archibald W., Hines, Edgar A., Jr., and Gage, Robert P., *Am. J. Dis. Child.*, 1945, **60**, 203.

<sup>5</sup> Downing, Elizabeth M., *Am. J. Dis. Child.*, 1947, **73**, 293.

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## Budding of Thrombocytes from Megakaryocytes.\* (17375)

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It is now generally agreed that the megakaryocyte is the source of the thrombocyte.<sup>1</sup> Dameshek and Miller<sup>2</sup> discuss the changes that occur in the marrow in idiopathic thrombocytopenic purpura. The latter authors found that, in the marrows of 10 normal subjects, an average of 68.6% of the megakaryocytes showed thrombocyte budding. Cartwright and associates<sup>3</sup> found thrombocyte budding in 75% of megakaryocytes in normal marrows. In a recent study of bone marrow films made by the citrate technic, as described below, from 12 patients with idiopathic thrombocytopenic purpura before operation, it was found that 0.14% of the megakaryocytes showed evidence of thrombocyte budding. Eighteen postoperative films, made at varying intervals after operation on 10 of these patients showed thrombocyte budding from 9.2% of megakaryocytes. When the bone marrows of 7 normal control patients

without hematological abnormality were examined, however, thrombocyte budding was seen in only 0.43% of the megakaryocytes. This discrepancy in the results obtained in the normal suggested that the differences might be due to variations in the technic used. Since the routine method for making bone marrow films in this Institute involves the use of a dry syringe, further investigations were carried out on material obtained here in this way.

*Methods. Citrate method.* The puncture of the sternum was performed with a Salah needle introduced at the level of the third or fourth intercostal space. The syringe and needle were first washed out with sterile 3.8% solution of sodium citrate, and aspirated material, usually 1 cc or less, was expressed on a watch glass which also had been rinsed with sodium citrate. Flecks of marrow were gently lifted with tweezers, and placed on carefully cleaned glass slides. They were then spread by means of another glass slide which was laid on top of the marrow flecks and slid rapidly across the surface of the first slide. The marrow films were dried in air. In the investigations referred to above, May-Grunwald-Giemsa stain had been used. In the investigations described below, Wright's stain was used, and the technic differed in that, following aspiration with a dry syringe, a sec-

\* The author is glad to acknowledge the assistance of Dr. C. C. Sturgis and Dr. F. H. Bethell in providing the facilities for the carrying out of this study.

† Rockefeller Travelling Research Fellow.

<sup>1</sup> Tocantins, L. M., *Medicine*, 1933, **17**, 155.

<sup>2</sup> Dameshek, W., and Miller, E. B., *Blood*, 1946, **1**, 27.

<sup>3</sup> Cartwright, G. E., Chung, Hui-Lan, and Chang, An, *Blood*, 1948, **3**, 249.

mens are shown on Table I.

Marrow aspirates obtained before and after splenectomy in four cases of idiopathic thrombocytopenic purpura were studied by the dry technic (Table II). In the peripheral blood, the thrombocytes were extremely few before operation. In 3 of the patients marrow films were made soon after splenectomy when the thrombocyte level was normal, and in the fourth case, the second puncture was made 3 years after operation at a time when the thrombocytes were recorded as being normal. Estimates of thrombocyte numbers rather than actual enumeration were employed because it is considered at this Institute that too much significance is apt to be attached to variations in the thrombocyte count which may, in fact, be due to technical factors or to differences in distribution of thrombocytes.

Sternal aspirations were performed on 5 patients with hematologic disorders and films were made by both dry and citrate methods. The results of the examination of the preparations are shown on Table III.

*Discussion.* There is obviously a very great difference between the results of thrombocyte counts on marrow aspirate obtained by the dry technic and that secured with the citrate method. It will be seen from Table III that there is less difference in areas where the marrow structure is preserved.

It would appear that when sodium citrate is used to flush out the syringe with which the sternal puncture is to be performed, many thrombocytes are removed from the marrow sample, and that this removal of thrombocytes is most marked in areas where the marrow structure is not intact. It might, therefore, be argued that the correct estimate of thrombocyte budding can be made only when a dry syringe is used. The figures given in Table III, however, indicate that even when this method is employed, the results are open to question, because here the estimates may be equally fallacious. When the thrombocyte count is high in the peripheral blood, one

would expect that their number would also be increased in the marrow and in the blood which inevitably dilutes the marrow sample. The thrombocytes are sticky, and under the conditions of sternal aspiration one would expect them to adhere to one another and to the megakaryocytes, the largest cells which they encounter. Hence, it would seem that counts performed with the dry technic are also inaccurate since much apparent thrombocyte budding is due to chance juxtaposition and adhesion of the sticky thrombocytes to the megakaryocyte. The estimate that is likely to be most valid is that made from areas with definite marrow structure. Even here, however, fields without megakaryocytes may contain an equal number of thrombocytes, and so it is not possible to say with certainty that in the normal marrow the number of megakaryocytes that show budding is as high as 25 per cent. Marrow structure is less well preserved when the citrate technic is used and it would appear that this method is unsatisfactory for investigations concerned with the budding of thrombocytes from megakaryocytes.

*Summary.* When marrow aspirations are performed using a syringe flushed with sodium citrate, a false impression is obtained of the amount of budding of thrombocytes from megakaryocytes because many thrombocytes are washed away from the parent cells.

When a dry technic is employed, the results are equally inaccurate because thrombocytes that by chance are in juxtaposition with megakaryocytes may stick to them and appear to be arising from them.

When aspirated marrow samples are used, the best indication of the extent of budding is obtained when a dry technic is used, and examination of the marrow is limited to areas of the film that have definite marrow structure. It is probable that less than twenty-five per cent of megakaryocytes in the normal marrow show true budding at any one time.

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TABLE III.  
Plate Patients Studied by Both the Dry and the Citrate Technique.

plate patients selected by blood film only and the culture technique							
	Platelets with megakaryocytes		Platelets without megakaryocytes		% of megakaryocytes		
	Thromb. per 100 megak.	Thromb. clumps per 100 meg.	Thromb. per 100 fields	Thromb. clumps per 100 fields	Thromb. per 100 fields	Thromb. clumps per 100 fields	% of megakaryocytes
Polycythemia anemia (thrombocytes normal)							
Dry method	248	4	556	4			20
Areas with structure	748	32	476	24			52
Areas with no structure							
Citrate method	120	nil	100	nil			15
Areas with structure	nil	nil	8	nil			0
Areas with no structure							
Polycythemia anemia (thrombocytes much reduced)							
Dry method	64	nil	20	nil			8
Areas with structure	332	nil	104	nil			60
Areas with no structure							
Citrate method	12	nil	4	nil			2
Areas with structure	nil	nil	nil	nil			0
Areas with no structure							
Chronic granulocytic leukemia (thrombocytes increased)							
Dry method	168	60	312	28			52
Areas with structure	1584	88	444	nil			92
Areas with no structure							
Citrate method	404	44	320	20			48
Areas with structure	76	12	56	4			16
Areas with no structure							
Chronic granulocytic leukemia (thrombocytes much increased)							
Dry method	32	72	64	32			68
Areas with structure	1066	212	1456	72			96
Areas with no structure							
Citrate method	550	110	60	20			70
Areas with structure	60	30	76	50			20
Areas with no structure							
Idiopathic thrombocytopenic purpura (platelets very much reduced)							
Dry method	4	nil	12	nil			0
Areas with structure	24	nil	4	nil			1
Areas with no structure							
Citrate method	nil	nil	nil	nil			0
Areas with structure	nil	nil	nil	nil			0
Areas with no structure							

TABLE I.  
Experiment with Selenium (Sodium Selenite, SS).<sup>\*</sup>  
Ten animals in each series.

Sex	Drug administered and method	Dosage (mg/kg)	Survival time (days)
♀	S.S.-(I.P.)	5	All survived
♂	"	5	" "
♀	"	10	Avg—10
♂	"	10	Avg— 6
Experiment with BAL (British Anti-Lewisite)			
♀	BAL-(I.M.)	15	All survived
♂	"	15	" "

\* Age of all rats used, 6 wk.

TABLE II.  
Experiment with BAL and Selenium.\*

No. of animals and sex	Drug administered and method	Dosage (mg/kg)		Survival time days (avg)
		BAL	SS	
8 ♀	BAL (I.M.) + SS (I.P.)	15	5	10
8 ♂	"	15	5	6
8 ♀	"	7.5	5	6
8 ♂	"	7.5	5	5
(Using same animal after 20 days on BAL).				
10 ♀	BAL (I.M.) + SS (I.P.)	15	5	3
10 ♂	"	15	5	3

\* Age of all rats used, 6 wk.

a survival time of  $5\frac{1}{2}$  days ranging from 5 to 6 days (Table II). Microscopic examination of the liver showed no pathology but the kidneys exhibited a tubular necrosis.

2. Pre-treatment with BAL. Rats were pretreated 20 days with BAL, 15 mg/kg. On the 21st day and days after for as long as they lived, 5 mg/kg of SS was administered simultaneously with 15 mg/kg of BAL. The average survival time in this combined treatment was 3 days (Table II). Microscopic examination of the liver was normal but the kidney again exhibited moderate necrosis (Fig. 2).

**Discussion.** When SS was used alone a typical toxic picture was noted.<sup>6</sup> The livers of these animals were necrotic and showed moderate to severe fatty degeneration.

BAL alone did not produce any untoward results in our experience although Durlacher *et al.*<sup>5</sup> reported toxic reactions in mammals from the doses we used. The results of the combined doses of BAL and selenium led to a marked decrease in the survival time of the animals treated. This finding corresponds to

the report of Braun, Lusky, and Calvery.<sup>4</sup> However, these investigators did not report any microscopic examination of the liver and kidney.

In our work, careful examination of the liver and kidney showed that, contrary to the usual findings in SS poisoning, there were no abnormalities in the liver tissue although the animals died rapidly. This might be regarded as due to the short survival time but it was noted that animals treated with SS alone for a period of 4 to 5 days *did* show the beginning of liver necrosis.

The results of our experiments would seem to indicate that the liver damage and death produced by SS may be entirely separate phenomena. As evidence in support of this contention BAL plus SS decreased the survival time of all animals as compared to those receiving SS alone. However, BAL protected SS animals from the liver damage usually produced by SS.

Seemingly, BAL does not antagonize the total toxicity of SS as it does other metals. However, it appears fair to propose that certain SH groups furnished by BAL prevent the liver damage caused by SS but are incapable

<sup>6</sup> Smith, M. I., Strohlman, E. F., and Lillie, R. D., *J. Pharm. and Exp. Therap.*, 1937, 60, 449.



## Administration of BAL\* in Selenium Poisoning.† (17376)

J. B. BELOGORSKY AND DONALD SLAUGHTER.

*From the Department of Physiology and Pharmacology, University of South Dakota School of Medicine, Vermillion.*

Selenium causes "alkali disease" or "blind staggers" in livestock in the Dakotas, Kansas, Montana, Wyoming, and other Western States where the soil and vegetation are high in selenium content. Therefore the toxic properties of selenium and its compounds are of great importance in these areas.

As far as we know, there is no reliable agent which is of value in preventing or curing the symptoms of selenium poisoning, though protective action of arsenic has been demonstrated by Moxon *et al.*<sup>1</sup>

Peters, Stocken, and Thompson<sup>2</sup> described the effectiveness of BAL in counteracting the effects of arsenical poisoning. The protective action of this compound in arsenical poisoning was believed to be due to the release of SH groups by BAL which could compete with the tissue SH groups for the arsenic, and act as a detoxifying agent. The nature of the reaction between arsenic and BAL made it seem probable that BAL would react similarly with other metals, and Gilman, Allen, and Phillips<sup>3</sup> have reported its effectiveness as an antidote for bichloride of mercury and cadmium poisoning, and Braun, Lusky, and Calvery<sup>4</sup> showed similar results against poisoning by antimony, bismuth, chromium and nickel. However, they demonstrated that lead and selenium were made more toxic by BAL.

\* BAL was kindly supplied by Hynson, Westcott and Dunning, Inc., of Baltimore, Md., and the Army Medical Center, Bethesda, Md.

† A preliminary report was presented at the Federation of American Societies for Experimental Biology in Detroit, Mich., April 18-22, 1949.

1 Rhian, M., and Moxon, A. L., *J. Pharm. and Exp. Therap.*, 1943, **78**, 249.

2 Peters, R. A., Stocken, L. A., and Thompson, R. H. S., *Nature*, 1945, **156**, 616.

3 Gilman, A., Phillips, F. S., Allen, R. P., Koelle, E. S., *J. Pharm. and Exp. Therap.*, 1946, **87**, 85.

4 Braun, H. A., Lusky, L. M., and Calvery, H. O., *J. Pharm. and Exp. Therap.*, 1946, **87**, 119.

Our experiments performed using BAL as an antagonist against selenium poisoning confirmed the results already described.<sup>4</sup> However, we have found no explanation in the literature of the apparent synergism between BAL and selenium, hence our work has been directed along this line.

*Experimental.* Healthy white rats 6 to 8 weeks old, 120 and 150 g of weight were used. Selenium was administered by intraperitoneal injections in the form of a freshly prepared aqueous solution of sodium selenite (SS). Intramuscular injections of BAL freshly dissolved in peanut oil were used. Each drug was administered daily for varying lengths of time.

A. *Toxicity of selenium.* The toxicity of selenium was determined by injections of (SS) in daily doses of 5 mg/kg of body weight. (This is equivalent to 2.2 mg of the element selenium). The animals were maintained on this regimen for a period of 28 days at which time they were sacrificed and the livers were examined for any possible pathology. All rats survived the full experimental period. (Table I). Microscopic examination showed a typical picture of liver necrosis and fatty degeneration (Fig. 1) as previously described by other workers.<sup>6</sup> Microscopic examination of the kidney was negative.

B. *Toxicity of BAL.* In this group all rats survived for the experimental period after receiving BAL in doses of 15 mg/kg of body weight. (Table I). Liver and kidney showed no abnormalities.

C. *Toxicity of SS and BAL.* 1. Combined treatment with BAL and SS. Rats injected simultaneously with BAL and selenium, 15 mg/kg and 5 mg/kg, respectively, had an average survival period of 8 days ranging from 6 to 10 days; those receiving doses of 7.5 mg/kg BAL and 5 mg/kg of selenium had

6 Durlacher, S. H., Bunting, H., Harrison, H. E., Ordway, N. K., and Albrink, W. S., *J. Pharm. and Exp. Therap.*, 1946, **87**, 28.

establishment of a 24-hour fast as the standard procedure, in which food is removed from the cage in the morning and the animals used the next morning. Considerable data have been accumulated on this preparation, and it will continue to be a very useful acute one. However, one great disadvantage of such a procedure is that it should not be repeated within a week if the animals are to be maintained in good condition. This restriction makes it impossible to study in each animal the time-curve of response to an experimental procedure and when used chronically over a period of weeks may seriously debilitate a high proportion of the rats.

Since rats learn readily, it was considered feasible to attempt to train them to consume their food from 5 to 10 P.M., so that they would be post-absorptive 15 hours later, 1 P.M. on the following day. Oxygen consumption or other metabolism studies could then be made from 1 to 5 p.m. without in any way interfering with the 5 to 10 feeding period for that day. The findings fulfilled the expectations.

**Experimental.** For most work in this laboratory, 4 adult rats are kept in a metal cage 20" x 16" x 12". The food, in checkers about 1" x 1" x 1/2" (Purina Dog Chow, Purina Laboratory Chow, Rockland Rat Diet), is fed from metal containers with the front and bottom out of 1/4" mesh hardware cloth. The animals have no trouble biting off pieces of about 1/8" to 3/8" in diameter, which they immediately eat from their own forepaws. These food checkers have also been ground to pass a 16 mesh screen and this fed from two open-top, cup-type containers per cage with equally satisfactory results. After removing the food containers, one should search the sawdust, paper strips, or other litter used on the floor of the cage for any stray pieces of food which could be consumed by the rats after the 10 P.M. deadline.

Oxygen consumption measurements were made in a modified Benedict<sup>1</sup> 4-unit closed-circuit apparatus.

**Results and discussion.** Twelve male and 12 female rats were placed on the feeding

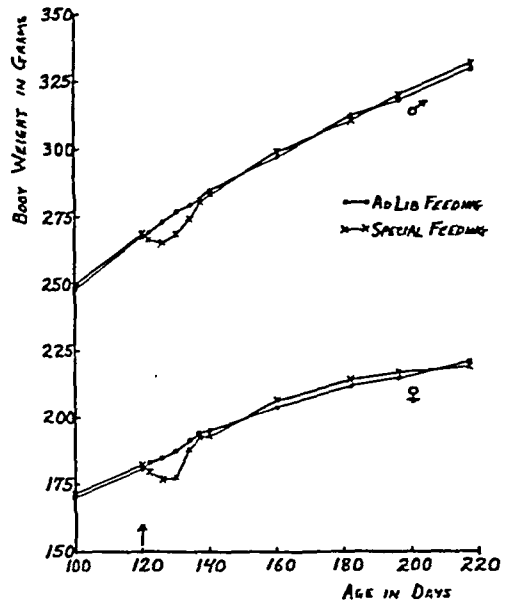


FIG. 1.

Body weight changes in rats placed on 5-hour feeding regime at 120 days of age.

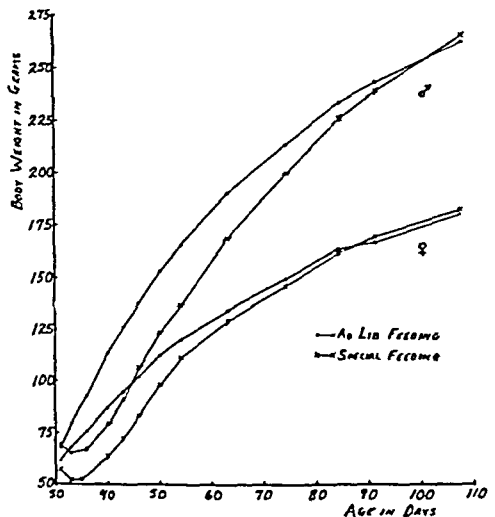


FIG. 2.

Growth curves of rats placed on 5-hour feeding regime upon weaning at 31 days of age.

routine outlined when 120 days of age, while an equal number of littermate controls were kept on *ad lib* feeding. Fig. 1 reveals that these young adult animals were able to maintain their body weights after a preliminary period of about 2 weeks for adjustment to the schedule. Body weight measurements are

<sup>1</sup> Benedict, F. G., *J. Nutrition*, 1930, 3, 161.

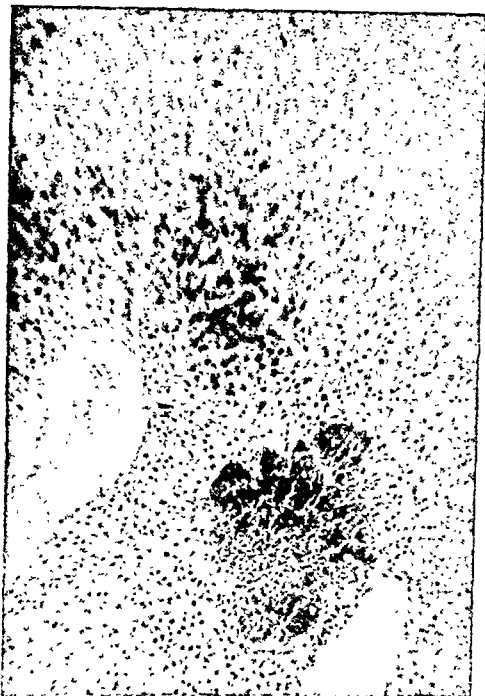


FIG. 1.

Rat liver: Typical focal necrosis and fatty degeneration following the administration of selenium in doses of 5 mg/kg for 10 days.

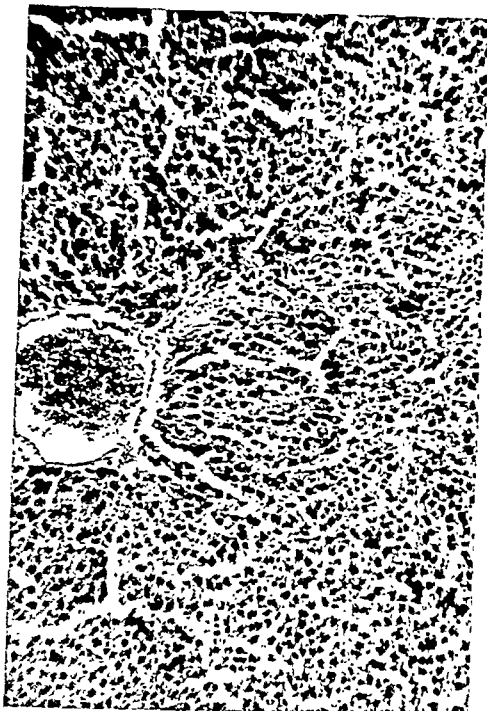


FIG. 2.

Rat liver: No abnormalities, following simultaneous administration of BAL 15 mg/kg and SS 5 mg/kg for 10 days.

of completely detoxifying the actions of SS.

It is now evident that BAL diminishes the toxicity of selenium for certain vital tissues and enhances the toxicity of the metal for the kidney. This corresponds to the findings of Gilman *et al.*,<sup>3</sup> who showed that cadmium becomes nephrotoxic with BAL. There are now

evidently two such metals that have this property.

Our thanks are due Doctor R. L. Ferguson, Professor of Pathology, for reporting the microscopic findings in this paper.

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### A Limited Feeding Regime for Rats.\* (17377)

S. B. BARKER.

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For the past 5 years, a rat feeding regime has been in use by this laboratory which has proved so helpful under varied circumstances

\* This investigation was supported in part by a grant from the Division of Research Grants and Fellowships, National Institutes of Health, U. S. Public Health Service.

as to warrant this brief presentation. In carrying out metabolic research, some baseline of activity is needed. The definition of the "post-absorptive condition" has met this need in the human and dog, essentially daytime animals. However, the nocturnal feeding habits of the laboratory rat have led to the

## Effect of Chronic Administration of Mercurial Diuretics on Glomerular Filtration in the Dog.\* (17378)

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The acute toxic effects of mercurial diuretics on the heart when administered intravenously are well known.<sup>1-4</sup> Recent reports on Thiomerin, the di-sodium salt of N( $\gamma$ -carboxymethylmercaptomercuri,  $\beta$ -methoxy) propyl camphoramic acid, have stressed the low cardiac<sup>5,6</sup> and local<sup>7</sup> toxicity when this mercurial diuretic is administered subcutaneously. Enthusiastic support has been given by several authors<sup>8-11</sup> for clinical use of this drug on cardiac patients.

In the following report, we have determined the effect of 3 mercurial diuretics, Thiomerin, meralluride (Mercurydrin), and mersalyl-theophylline (Merthyl) on glomerular filtration rate in the dog when these compounds are administered intravenously in comparable daily doses.

*Methods.* Control creatinine clearances were

determined on each trained unanesthetized adult female dog one hour after hydration with 40 cc/kg of water by stomach tube. Creatinine determinations were performed by the method of Folin and Wu.<sup>12</sup> A series of 10 dogs were tested with Thiomerin, nine with Mercurydrin, and 5 dogs were given Merthyl. The diuretics were administered intravenously in a dosage of 0.1 cc/kg/day (0.39 mg Hg/kg/day) for a period of 21 to 28 days. Creatinine clearances were determined at weekly intervals during the test period and in some instances were continued for one or two weeks after drug administration had been discontinued. In addition, mercury excretion determinations were made on three dogs of the Thiomerin series.

*Results.* Comparison was made of weekly changes in creatinine clearances of the dogs in each drug series. These changes were calculated in terms of percentage increase or reduction in the weekly creatinine clearance of each dog as compared to its control clearance level (Fig. 1, 2 and 3). It was observed that the creatinine clearances in many of the test animals were significantly reduced at the end of the third week of drug administration. However, there was noted a distinct trend toward return to the control clearance level during the recovery period in those animals which were followed for one or two weeks after the drug administration was discontinued.

Comparing the mean percentage changes from the control clearances of the dogs in each series indicates that in the third week Thiomerin produced a significantly greater reduction of creatinine clearance than the other mercurial diuretics tested (Fig. 4).

Evidence of toxicity from the mercurial diuretics developed early in some of the test animals. Three dogs in the Thiomerin series

\* This investigation was supported in part by a grant from The Lakeside Laboratories, Inc.

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<sup>1</sup> DeGraff, A. C., and Lehman, R. A., *J.A.M.A.*, 1942, **119**, 998.

<sup>2</sup> Volini, I. F., Levitt, R. O., and Martin, R., *J.A.M.A.*, 1945, **128**, 12.

<sup>3</sup> Long, W. K., and Farah, A., *J. Pharm. and Exp. Therap.*, 1946, **88**, 388.

<sup>4</sup> Lehman, R. A., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 428.

<sup>5</sup> Lehman, R. A., and King, E. E., *Fed. Proc.*, 1949, **8**, 314.

<sup>6</sup> Herrmann, G. R., Chriss, J. W., Schwab, E. H., Hejtmancik, M. R., and Sims, P. M., *Fed. Proc.*, 1949, **8**, 74.

<sup>7</sup> Lehman, R. A., Taube, H., and King, E. E., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 1.

<sup>8</sup> Ruskin, A., Johnson, J. E., and Roddy, W. N., *Fed. Proc.*, 1949, **8**, 329.

<sup>9</sup> Grossman, J., Weston, R. E., Edelman, I. S., and Letter, L., *Fed. Proc.*, 1949, **8**, 62.

<sup>10</sup> Batterman, R. C., Unterman, D., and DeGraff, A. C., *Fed. Proc.*, 1949, **8**, 272.

<sup>11</sup> Herrmann, G. R., *J.A.M.A.*, 1949, **140**, 509.

<sup>12</sup> Folin, O., and Wu, H., *J. Biol. Chem.*, 1919, **38**, 81.

TABLE I.  
Metabolic Rates of Rats Fasted 24 Hours and of  
Rats on Special Feeding Regime.

	cc O <sub>2</sub> /100 g/hr	
	24-hr fast	special regime
28 ♂	100.9	101.3
20 ♀	102.8	102.7
Avg of all 48	101.7 ± 4.66*	101.9 ± 4.82*

\* Standard deviation.

probably more accurate when taken in the afternoon on this regime than on *ad lib* feeding, since occasional animals in the latter group eat during the daytime.

Even weanlings placed on the 5-hour feeding can eventually match the growth curves of littermates fed *ad lib*, as is shown in Fig. 2. In this experiment, groups of eight rats were weaned at 31 days of age from their mothers to ground rat food, available either *ad lib* or between 5 and 10 P.M. In these instances, the considerably longer time of nearly 2 months was required for complete readjustment, probably because of the increased requirements imposed by growth. Similar data are available for weanlings given their food in checker form.

Metabolic rates were compared in 48 adult rats, first after the conventional 24-hour fast, and then 3 to 6 weeks after being transferred to the limited feeding period regime. Table I shows that the two sets of oxygen consumption data were essentially identical. No apparatus was available for determining CO<sub>2</sub> production, so no R.Q.'s have been obtained for comparison. One would anticipate lower values after the extra 9 hours without food.

Although not shown because the results

were not significantly different from those already discussed, 24 adult rats were trained to eat from noon to 5 P.M. so as to be ready for metabolism determinations at 8 A.M. on the next day. Even this remarkable alteration of habits, which essentially made daytime animals out of nocturnal ones, was successfully met.

Tepperman *et al.*<sup>2</sup> and Dickerson *et al.*<sup>3</sup> have shown that shortening the daily feeding time of rats to one hour resulted in such a distorted appetite in relation to caloric needs that the animals became obese. No such alteration in eating pattern has ever been observed in our animals. One may conclude from this that 5 hours' feeding time places no strain on the rat's ability to satisfy its appetite. No attempt was made to determine whether any of the alterations in carbohydrate metabolism noted by these authors could be shown to exist in our animals.

*Summary.* It has been found possible to train rats to eat their usual amounts of food in a 5-hour period, between 5 and 10 P.M. Fifteen hours later, at 1 P.M. on the following day, the animals are in a post-absorptive condition, prepared for a variety of metabolism studies.

The animals can be maintained indefinitely on this regime and the debilitating results of repeated periods of fasting for 24 hours can be avoided.

<sup>2</sup> Tepperman, J., Brobeck, J. R., and Long, C. N. H., *Yale J. Biol. Med.*, 1943, 15, 855.

<sup>3</sup> Dickerson, V. C., Tepperman, J., and Long, C. N. H., *Yale J. Biol. Med.*, 1943, 15, 875.

THE EFFECTS OF MERCURIAL DIURETICS ON GLOMERULAR FILTRATION

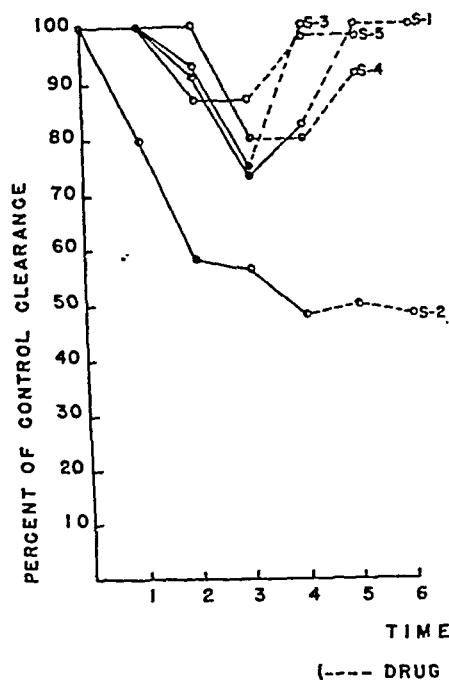


FIGURE 3 MERTHYL

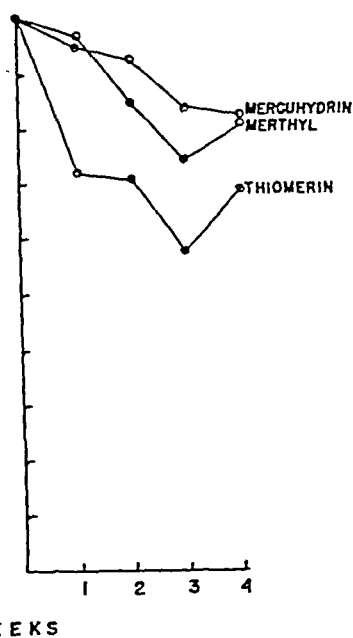


FIGURE 4 MEAN CLEARANCE REDUCTIONS

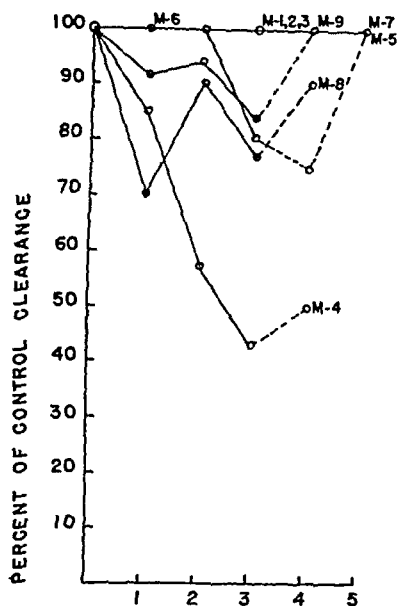
in the animals given the other two drugs. We also noted that by the third week of drug administration, the creatinine clearances of the Thiomerin dogs were reduced to a greater extent than those of the dogs in the Mercurhydrin and Merthyl series. Special care was taken to use solutions of Thiomerin which were kept refrigerated and not over 4 days old.

Data obtained from mercury excretion determinations of 3 Thiomerin dogs suggested that the excretion of the drug is not as rapid or complete as with other mercurial diuretics. In the few studies conducted, the dogs that showed symptoms of mercury poisoning also showed a delayed excretion of mercury.

*Summary.* Mercurial diuretics administered intravenously in chronic dosage to dogs reduced the glomerular filtration rate as measured by the creatinine clearance. This reduction in glomerular filtration rate was found to be temporary in most dogs that did not have severe symptoms of mercury poisoning.

Thiomerin, when administered intravenously in comparable chronic dosage to the dog, was found to be of higher toxicity than either Mercurhydrin or Merthyl, as indicated by reduced creatinine clearances, and other signs of mercury poisoning.

## THE EFFECTS OF MERCURIAL DIURETICS ON GLOMERULAR FILTRATION



TIME IN WEEKS  
(--- DRUG DISCONTINUED)

FIGURE 1 MERCURHYDRIN

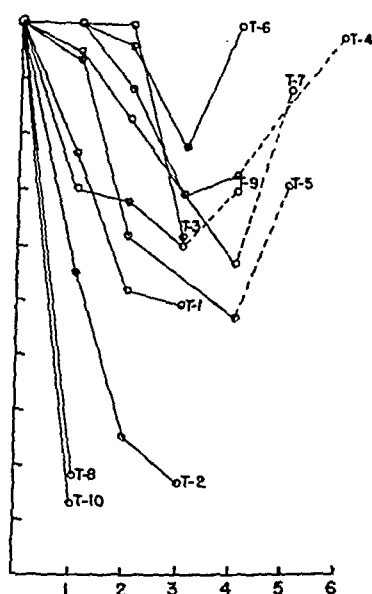


FIGURE 2 THIOMERIN

(T-3, T-8, and T-10) developed a severe stomatitis in the first week of drug administration. Stomatitis did not occur in the dogs of the Mercurhydrin or Merthyl series. Moderate to severe diarrhea occurred in 4 Thiomerin dogs (T-4, T-5, T-8 and T-10) and none appeared in the Mercurhydrin and Merthyl dogs. Severe dermatitis with generalized loss of hair developed in two Thiomerin dogs (T-6 and T-7) and in one Mercurhydrin dog (M-8). One Thiomerin dog, not included in the series, died on the seventh day and two other Thiomerin dogs (T-8 and T-10) died on the 8th day with evidence of severe mercurial poisoning. A fourth Thiomerin dog (T-6) died with acute pulmonary edema four days after the drug was discontinued. One of the Mercurhydrin dogs (M-6) died on the seventh day of apparent acute cardiac toxicity immediately following intravenous injection of the drug. No deaths occurred in the Merthyl series.

*Discussion.* The dose employed in these ex-

periments was chosen because it gives satisfactory diuresis in the dog for a period of 4-6 hours. When administered subcutaneously, a variable amount of mercury may be retained at the site of injection by local tissue combination, therefore the diuretics were given intravenously.

In general, evidence of mercurial poisoning was accompanied or heralded by an early reduction of the creatinine clearance. It would appear that the reduction in the glomerular filtration rate is a temporary manifestation if the drug is discontinued before irreparable renal damage is produced. In the dogs that had only mild signs of mercury poisoning, the creatinine clearance returned to normal one or two weeks after discontinuing the drug.

It appears that Thiomerin is more toxic than either Mercurhydrin or Merthyl when tested in this manner. Our opinion is based on the observation of evidence of mercury poisoning such as stomatitis and diarrhea which occurred more frequently in the Thiomerin dogs than

TABLE I.  
Survival of Malaria Sporozoites in Various Whole Blood, Plasma and Cell Mixtures.

Mixtures of canary and hen whole bloods (C = canary, H = hen).			
Trial No.	1/10 C-9/10 H (birds infected)	5/10 C-5/10 H (birds infected)	9/10 C-1/10 H (birds infected)
1	1/4	2/3	4/4
2	3/5	5/5	5/5
3	2/5	4/4	5/5
4	1/5	3/5	3/5
5	0/8	7/8	8/8
6	1/8	1/8	5/7
7	4/8	7/7	6/7
Totals	12/43 (27%)	29/40 (72%)	36/41 (87%)
Canary and hen plasmas alone.			
Trial No.	C plasma (birds infected)	H plasma (birds infected)	
1	0/12	0/12	
2	0/12	0/12	
Totals	0/24	0/24	
Canary plasma alone and with Canary cells.			
Trial No.	Alone (birds infected)	With C cells (birds infected)	
1	0/6	6/6	
2	0/6	6/6	
3	0/6	6/6	
4	0/6	6/6	
Totals	0/24 (0%)	24/24 (100%)	
Canary plasma with Canary cells, Hen cells, and both.			
Trial No.	With C cells (birds infected)	With H cells (birds infected)	With C and H cells (birds infected)
1	5/5	0/5	4/5
2	5/5	0/5	2/5
3	5/5	0/5	1/5
4	4/4	0/4	4/4
5	8/8	0/7	8/8
6	7/7	0/7	6/8
7	8/8	0/8	7/8
Totals	42/42 (100%)	0/41 (0%)	32/43 (74%)
Hen plasma with Canary cells, Hen cells, and both.			
Trial No.	With C cells (birds infected)	With H cells (birds infected)	With C and H cells (birds infected)
1	4/4	0/4	4/4
2	4/4	0/4	4/4
3	4/4	0/4	1/4
4	4/4	0/4	4/4
5	4/4	0/4	4/4
Totals	20/20 (100%)	0/20 (0%)	17/20 (85%)
Canary plasma with Canary white and red cells, separately.			
Trial No.	White cells (birds infected)	Red cells (birds infected)	Plasma alone (birds infected)
1	5/5	3/5	0/5
2	5/5	3/5	0/5
3	5/5	3/5	1/5
4	8/8	3/8	0/8
5	5/7	1/8	0/8
Totals	28/30 (93%)	13/31 (41%)	1/31 (3%)

half of which was canary plasma and half Locke's solution containing the sporozoites. One small drop of red cells obtained from deep down in the bulb of the pipette was then



# Influence of Cells and Plasma on *In vitro* Survival of Malaria Sporozoites.\* (17379)

HARRY BECKMAN.

*From the Department of Pharmacology, Marquette University School of Medicine, Milwaukee.*

It has been shown that the sporozoites of *Plasmodium cathemerium* 3H2, infective for the canary but not the hen, retain their infectivity during several hours in drawn canary but not drawn hen blood.<sup>1</sup> The present communication reports investigation of the effect of the cell and plasma portions of these bloods upon the retention of infectivity.

*Mixtures of canary and hen whole bloods.* Infected mosquitoes were ground in Locke's solution and added to the following freshly prepared mixtures: (a) one-tenth canary and nine-tenths hen blood; (b) five-tenths canary and five-tenths hen blood; (c) nine-tenths canary and one-tenth hen blood. After incubation of the blood-sporozoite mixtures with gentle agitation at 41.5°C for 1½ hours, portions representing one mosquito were injected intramuscularly into canaries, the peripheral blood of these birds being subsequently examined from the 5th to 16th days, inclusive. Birds failing to become positive were proved negative in all instances by subsequent infection from mosquito bites. Trials were made at such intervals that in most instances at least one insectary cycle had been completed between them.

In the trials employing mixture (a), 27% of the birds became infected (see Table I); mixture (b) infected 72% and mixture (c) 87%.

*Canary and hen plasmas alone.* Sporozoites were separately incubated in canary and hen plasmas. In two trials (See Table I), they failed to retain infectivity in either plasma.

*Canary plasma alone and with canary cells.* Four trials were made of sporozoites in canary plasma alone and in another portion of the same plasma to which the normal amount of canary cells was returned after 3 washings in

Locke's solution. None of the sporozoites retained their infectivity in the plasma alone (see Table I), but they did so in 100% of instances when the cells were present.

*Canary plasma with canary cells, hen cells, and both.* Seven trials were made of sporozoites in canary plasma to which the washed canary cells were returned, canary plasma to which an equivalent amount of washed hen cells were added, and canary plasma in which one-half the added washed cells were canary and the other half hen. When canary cells alone had been added, sporozoite infectivity was retained in 100% of instances (see Table I); when hen cells alone had been added, infectivity was lost completely; when the canary-hen cell mixture had been added, infectivity was retained to the extent of 74%.

*Hen plasma with canary cells, hen cells, and both.* These 5 trials constituted a test just the reverse of the above in that the fluid medium was hen instead of canary plasma. When hen cells were added there was no retention of sporozoite infectivity (Table I); when canary cells were added infectivity was retained 100%; when a mixture in equal parts of the two types of cells was added infectivity was 85% retained.

*Canary plasma with canary white and red cells, separately.* In 5 trials, after the initial separation of canary plasma and cells by centrifugation, the cells were washed 3 times in Locke's solution and then loaded through a long needle into the bulb and stem of a 2 cc Ostwald-Folin pipette modified by cutting off the delivery tip below the bulb, fusing the lower opening, and cutting down the suction stem to a size suited to the centrifuge. Spinning of this improvised centrifuge tube loaded with washed total blood cells delivered the white cells as "buffy layer" into the suction stem, whence they were removed with a thinly drawn capillary tube. One small drop of this buffy layer was blown into a 0.6 cc mixture,

\* Research supported by National Institute of Health Research Grant 67 (C2).

<sup>1</sup> Beckman, H., *Proc. Soc. Exp. Biol. and Med.*, 1948, 67, 172.

TABLE I.  
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2	0/6	6/6	
3	0/6	6/6	
4	0/6	6/6	
Totals	0/24 (0%)	24/24 (100%)	
Canary plasma with Canary cells, Hen cells, and both.			
Trial No.	With C cells (birds infected)	With H cells (birds infected)	With C and H cells (birds infected)
1	5/5	0/5	4/5
2	5/5	0/5	2/5
3	5/5	0/5	1/5
4	4/4	0/4	4/4
5	8/8	0/7	8/8
6	7/7	0/7	6/8
7	8/8	0/8	7/8
Totals	42/42 (100%)	0/41 (0%)	32/43 (74%)
Hen plasma with Canary cells, Hen cells, and both.			
Trial No.	With C cells (birds infected)	With H cells (birds infected)	With C and H cells (birds infected)
1	4/4	0/4	4/4
2	4/4	0/4	4/4
3	4/4	0/4	1/4
4	4/4	0/4	4/4
5	4/4	0/4	4/4
Totals	20/20 (100%)	0/20 (0%)	17/20 (85%)
Canary plasma with Canary white and red cells, separately.			
Trial No.	White cells (birds infected)	Red cells (birds infected)	Plasma alone (birds infected)
1	5/5	3/5	0/5
2	5/5	3/5	0/5
3	5/5	3/5	1/5
4	8/8	3/8	0/8
5	5/7	1/8	0/8
Totals	28/30 (93%)	13/31 (41%)	1/31 (3%)

half of which was canary plasma and half Locke's solution containing the sporozoites.

One small drop of red cells obtained from deep down in the bulb of the pipette was then

blown into another portion of the same plasma-Locke-sporozoite mixture. Incubation and subsequent injections as usual.

Survival of sporozoite infectivity occurred in 93% of instances when white cells alone had been returned to the plasma (see Table I); in 41% when red cells alone had been added; in 3% when presumably plasma alone was present.

*Discussion.* In the recent interesting paper of McGhee,<sup>2</sup> who succeeded in infecting Swiss mouse erythrocytes injected intravenously into chick embryos 2 days after infecting the embryos intravenously with *P. lophurae* trophozoites, it is suggested that perhaps the malaria resistant principle in certain species lies in the serum constituents rather than in the cells. The studies herein reported indicate that the reverse is the case with regard to sporozoite survival, at least in drawn blood. The sporozoites in these experiments did not survive in the plasma of either the resistant or the susceptible species, but they survived equally well in the plasma of either species provided cells of the susceptible species were present. They did not survive, however, in the plasma of the susceptible species if cells of the resistant species alone were added, though they survived extremely well in the plasma of either species if, in addition to cells of the resistant species, cells of the susceptible species were present. Apparently, therefore, insofar as one can draw conclusions from a study of two species of the same phylum, the survival of sporozoites in drawn blood depends upon the presence therein of cells of the susceptible species. This would appear to conform to the *in vivo* observation of Huff and Coulston,<sup>3</sup> who were unable to prevent sporozoite infection of *P. gallinaceum*-susceptible chickens by inoculation of them with the whole blood, serum or plasma of non-susceptible ducks, but who did succeed in conveying to ducks some of the susceptibility of the chicken by inoculating them with chicken blood.

Whether it is the white or red blood cells of the susceptible species which make survival possible was not conclusively determined in these studies, though the evidence (Table I) favors the greater importance of the white cells. If a technic can be evolved which will deliver pure samples of white and red cells, it should be possible to settle the point. Contamination of the white cells with a small number of red cells would not probably be important in view of the evidence, but it appears that the presence of only a few white cells in the red cell sample could considerably facilitate survival because it required only one small drop of the buffy layer in 0.6 cc of diluent to enable practically complete survival to take place.

No evidence of the nature of the action of the canary cells in facilitating sporozoite survival *in vitro* was obtained. Prolonged study of stained smears made at various times during the incubation of the mixtures did not reveal sporozoites or anything resembling pre-erythrocytic forms in any of the cells. Immunologic studies were not performed.

*Summary.* In a study of the role of cells and plasma in retention of infectivity of sporozoites of *P. cathemerium* 3H2 in canary blood and the failure of such retention in hen blood, it was found that (a) survival occurs in hen whole blood provided a small amount of canary whole blood is present; (b) it does not occur in either canary or hen plasma alone; (c) it occurs in either canary or hen plasma if canary cells are present whether or not hen cells are simultaneously present but does not occur in either plasma if only hen cells are present; (d) it occurs in canary plasma to a greater extent if canary white cells (somewhat contaminated with canary red cells) are present than if canary red cells (somewhat contaminated with white cells) are present. The possible significance of the findings is discussed.

Technical assistance of Darrell B. Paul is gratefully acknowledged.

<sup>2</sup> McGhee, R. B., PROC. SOC. EXP. BIOL. AND MED., 1949, **71**, 92.

<sup>3</sup> Huff, C. G., and Coulston, F., J. Infect. Dis., 1946, **78**, 99.

## Toxicity of Sodium Tetrathionate. (17380)

NATHAN BLOOM, GUS FORBES, AND LEONARD POLICOFF.  
(Introduced by H. B. Haag.)

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Sodium tetrathionate monohydrate ( $\text{Na}_2\text{S}_4\text{O}_6 \cdot \text{H}_2\text{O}$ ) was first proposed by Theis and Freeland<sup>1</sup> for use in the treatment of thrombo-angiitis obliterans. Their object was to provide a greater and more prolonged effect than that obtained by sodium thiosulfate ( $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ ). These substances comprise an oxidation-reduction system but thiosulfate will not oxidize SH groups while tetrathionate does have this action. It has been suggested by Phillips *et al.*,<sup>2</sup> Goffart and Fischer<sup>3</sup> that the kidneys may be damaged by this fundamental reaction.

The Council on Pharmacy and Chemistry of the American Medical Association<sup>4</sup> quoted the work of Chen, Rose, and Clowes,<sup>5</sup> also that of Gilman and his associates,<sup>6</sup> on the toxicity of this substance. It was stated that the intravenous  $\text{LD}_{50}$  in the dog was 250 mg per kilo and in the rabbit 75 mg per kilo. The animals died from renal failure due to severe necrotizing lesions in the proximal convoluted tubules. A later report of the Council consisted of communications from Theis and DeTakats,<sup>7</sup> who emphatically stated that the clinical dose of sodium tetrathionate was only a small fraction of the lethal dose in animals. They advocated a review of the toxicity of this drug from a clinical standpoint.

In an effort to determine the toxicity of

sodium tetrathionate (Tetrathione, Searle) clinically, we decided on the following experiment.

*Method of study.* A group of 30 patients, admitted to the Medical Service of the Medical College of Virginia, Hospital Division, some with peripheral vascular disease, and others with various other maladies, were subjected to routine blood non-protein nitrogen, Mosenthal concentration and phenolsulphonphthalein tests. These patients were then given 0.6 g (10 cc) of tetrathione, intravenously twice daily, for a period of 7 days. This dosage was equivalent to that of the usual course of treatment of peripheral vascular disease, lasting 7 weeks. At the end of the 7 day period, the non-protein nitrogen, Mosenthal concentration, and phenolsulphonphthalein studies were repeated. The results are given in Table I.

There were no untoward reactions to the drug. None of the patients manifested nausea, vomiting, abdominal pain, or weakness, which have been reported following too rapid injection or during the course of clinical treatment. The changes in the laboratory findings were mostly of a minimal character. In 15 cases the non-protein nitrogen showed a slight increase, in 8 cases there was a slight decrease, and in 7 cases there were no changes. The Mosenthal concentration test showed an increase in specific gravity in 10 cases, a decrease in 7 cases, and in 13 cases there were no changes. The phenolsulphonphthalein test showed an increased concentration of the dye in 6 cases, in 15 cases there was less concentration, and in 9 cases there were no changes. The maximum increase in the non-protein nitrogen was from 20 mg% to 35 mg% in case 18, a patient with thrombo-angiitis obliterans. We consider 35 mg % within the normal range. The Mosenthal concentration test in case 6 showed a variation from a maximum specific gravity of 1.032 prior to tetrathione to 1.018 follow-

<sup>1</sup> Theis, F. F., and Freeland, M. R., *Arch. Surg.*, 1940, **40**, 190.

<sup>2</sup> Phillips, F. S., Gilman, A., Koelle, E. S., and Allen, R. P., *J. Biol. Chem.*, 1947, **167**, 209.

<sup>3</sup> Goffart, M., and Fischer, P., *Archives Internationales de Physiologie*, 1948, **55**, 258.

<sup>4</sup> Council on Pharmacy and Chemistry, *J.A.M.A.*, 1947, **133**, 693.

<sup>5</sup> Chen, K. K., Rose, C. L., and Clowes, G. H. A., *Am. J. Med. Sci.*, 1937, **183**, 767.

<sup>6</sup> Gilman, A., Phillips, F. S., Koelle, E. S., Allen, R. P., and St. John, E., *Am. J. Physiol.*, 1946, **147**, 115.

<sup>7</sup> Supplemental Report on Sodium Tetrathionate, *J.A.M.A.*, 1947, **134**, 1092.

blown into another portion of the same plasma-Locke-sporozoite mixture. Incubation and subsequent injections as usual.

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No evidence of the nature of the action of the canary cells in facilitating sporozoite survival *in vitro* was obtained. Prolonged study of stained smears made at various times during the incubation of the mixtures did not reveal sporozoites or anything resembling pre-erythrocytic forms in any of the cells. Immunologic studies were not performed.

**Summary.** In a study of the role of cells and plasma in retention of infectivity of sporozoites of *P. cathemerium* 3H2 in canary blood and the failure of such retention in hen blood, it was found that (a) survival occurs in hen whole blood provided a small amount of canary whole blood is present; (b) it does not occur in either canary or hen plasma alone; (c) it occurs in either canary or hen plasma if canary cells are present whether or not hen cells are simultaneously present but does not occur in either plasma if only hen cells are present; (d) it occurs in canary plasma to a greater extent if canary white cells (somewhat contaminated with canary red cells) are present than if canary red cells (somewhat contaminated with white cells) are present. The possible significance of the findings is discussed.

Technical assistance of Darrell B. Paul is gratefully acknowledged.

<sup>2</sup> McGhee, R. B., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 92.

<sup>3</sup> Huff, C. G., and Coulston, F., *J. Infect. Dis.*, 1946, **78**, 99.

enthal concentration values indicated little disturbance in kidney function. However, these tests would not be expected to show much change unless there was a rather extensive impairment in renal function. The phenolsulphonphthalein excretion test was our most sensitive test of tubular function and in several instances showed evidence of dimin-

ished tubular capacity following sodium tetrathionate administration. On the basis of these clinical studies it would seem that sodium tetrathionate probably should not be used if there is any pre-existing renal disease.

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### Glycogen in Basophilic Leucocytes in Human Blood Smears. (17381)

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In a recent study of the occurrence of the periodic acid-Schiff reaction in various normal cells of the blood and connective tissue by Wislocki, Rheingold and Dempsey,<sup>1</sup> small clear red dots were observed in the pale pink cytoplasm of the basophilic leucocytes. These investigators were not able to identify the basophiles in control blood smears digested by saliva. They therefore concluded that the red stained material in the undigested smears may have been glycogen but that the "apparent finding needs further verification." Similarly, Gibb, and Stowell<sup>2</sup> decided that "Because myeloid cells which were free from glycogen were not observed in normal peripheral blood and bone marrow films, basophils, although not specifically identified, may also contain glycogen."

To make certain that basophils could be identified in smears digested by saliva before treatment with periodic acid and leucofuchsin, dried smears of human blood were stained in Wright's blood stain in absolute methyl alcohol for 3 minutes. This step was not followed by the usual one of the diluted stain. For the most part, the smears were very quickly rinsed in 95% ethyl alcohol although at the beginning of the study a few were dipped in running water. Basophils were located and

each cell was ringed by means of an object marker. A map was made giving its position in the circle. The smears were then fixed in absolute ethyl alcohol for 5 minutes, rinsed in 95% and 70% alcohol and stained by the periodic-leucofuchsin technic of McManus<sup>3</sup> with  $\frac{1}{2}$ % periodic acid, or, as modified by Wislocki, Rheingold and Dempsey,<sup>1</sup> with 1% periodic acid. The smears to be digested were covered with saliva for 45 to 60 minutes after fixation in absolute alcohol and rinsed in running water. After the smears had been examined with no nuclear stain, they were stained in Mayer's hematoxylin for 10 minutes.

In the undigested smears, the glycogen in the neutrophiles was stained a deep to pale red-pink, either evenly throughout the cytoplasm, or more deeply either around the periphery of the cell or off at one side. The 29 basophilic leucocytes examined were, in contrast, much paler, a difference seen clearly when the two kinds of cells were found next to each other. There was a marked variation in the amount of glycogen in the basophiles even on the same slide. In some cells rather large clear red granules were seen in groups with finer grains distributed more regularly among the unstained basophilic granules. In others only these dust-like particles of glycogen were visible. A third variety had a hazy pink background which outlined the colorless basophilic granules. A few had no color at all.

<sup>1</sup> Wislocki, G. B., Rheingold, J. J., and Dempsey, E. W., *Blood*, 1949, 4, 562.

<sup>2</sup> Gibb, R. P., and Stowell, R. E., *Blood*, 1949, 4, 569.

<sup>3</sup> McManus, J. F. A., *Stain Tech.*, 1948, 23, 99.

TABLE I.  
 Sodium Tetrathionate Experiments.

Case No.	P.S.P. control, %	P.S.P. after, %	N.P.N. control, mg %	N.P.N. after, mg %	Mosenthal (highest) Control	After
1	80	63	24	32	1.022	1.022
2	85	75	23	35	1.024	1.020
3	60	55	36	27	1.022	1.020
4	55	55	28	38	1.022	1.025
5	70	60	33	32	1.022	1.020
6	60	70	30	33	1.032	1.018
7	58	50	26	30	1.020	1.018
8	55	55	25	30	1.018	1.016
9	60	55	35	35	1.018	1.018
10	45	50	30	27	1.020	1.020
11	60	60	44	40	1.015	1.016
12	83	65	23	27	1.013	1.016
13	60	53	29	27	1.017	1.018
14	90	62	23	27	1.020	1.018
15	55	55	32	27	1.022	1.022
16	55	55	41	32	1.017	1.016
17	75	60	33	29	1.026	1.026
18	51	34	20	35	1.016	1.023
19	70	60	25	27	1.012	1.012
20	53	50	24	24	1.012	1.017
21	47	45	30	33	1.012	1.012
22	50	45	31	36	1.016	1.015
23	40	45	36	36	1.012	1.021
24	50	58	33	30	1.021	1.018
25	105	55	27	26	1.018	1.016
26	75	70	26	37	1.012	1.019
27	60	60	32	30	1.022	1.022
28	70	60	47	50	1.020	1.022
29	Incomplete					
30	40	45	36	37	1.014	1.012
Avg	63 $\pm$ 2.7	57 $\pm$ 1.7	30 $\pm$ .11	32 $\pm$ 10	1.018 $\pm$ .009	1.019 $\pm$ .0006

ing the drug. In all other cases the variation in Mosenthal concentration was minimal in either direction. The phenolsulphonphthalein test showed the most marked variations. It was decreased by 18% in case 12 and decreased by 27.5% in case 14. The most unusual change was in case 25, in which there was a decrease of 50%. There was no correlation in any separate case between the changes in the various tests.

A statistical analysis<sup>8</sup> of the data is outlined in Table I. It will be noted that the phenolsulphonphthalein control excretion  $63.1\% \pm 2.75$ . After sodium tetrathionate the excretion was  $56.8\% \pm 1.69$ . The "t" value for the difference of these means is 1.955 which, with a normal deviate of 1.96, gives a probability of 0.05. The values for the non-protein nitrogen before and after sodium tetrathionate were  $30.3 \text{ mg}\% \pm .115$ , and  $32.1 \text{ mg}\%$

$\pm 0.97$ . This indicates no significant difference. The Mosenthal values consider only the highest concentration obtained. They were  $1.018 \pm 0.009$  and  $1.019 \pm 0.0006$ . This indicates very little difference. The statistical analysis demonstrates that for the dosage employed sodium tetrathionate does not materially affect kidney function as regards the non-protein nitrogen and Mosenthal tests. The phenolsulphonphthalein excretion might be interpreted as definitely reflecting some changes in kidney function.

*Summary.* Thirty patients were chosen at random from the routine admissions to the Medical Service of the Medical College of Virginia Hospital Division and given 0.6 g sodium tetrathionate twice daily for 7 days, intravenously. The effect on kidney function was studied by following changes in blood non-protein nitrogen, Mosenthal concentration and phenolsulphonphthalein excretion values. The blood non-protein nitrogen and the Mos-

<sup>8</sup> Snedecor, G. W., *Statistical Methods*, fourth edition, Iowa State College Press, 1946.

**Coagulase.** Cell-free coagulase was prepared as follows: From 5-10 ml of an overnight culture of a coagulase-positive strain of *S. aureus* in tryptose broth were placed in a flask containing 500 ml of tryptose broth to which 10 ml of bovine albumin<sup>†</sup> had been added. This broth culture was incubated for 72 hours at 37°C, following which the cells were sedimented in a refrigerated centrifuge for 2 hours at 2,000 RPM. The supernate was removed and stored at 4°C overnight. The supernate was then passed through a Seitz filter, the first 50 ml being discarded. The filtrate was tested for sterility and stored in the frozen state in ampules. At the time of use it was thawed rapidly and the necessary dilutions made with sterile tryptose broth.

**Fibrinogen.** For standardization of coagulase a solution of 100 mg of Fraction I (bovine fibrinogen<sup>‡</sup>) in 63 ml of buffered saline solution was employed. After incubation at 37°C for 30 minutes with occasional agitation, 1 ml of the 1:100 dilution of heparin was added.

To 50 ml of this solution was added 0.1 ml of the standard activator. Using volumetric pipettes, this was further diluted with the fibrinogen solution so that the final concentration of activator was 1:3500.

For the coagulase-inhibition test, 100 mg of fibrinogen was dissolved in 31 ml of buffer and following incubation at 37°C for 30 minutes, 1 ml of 1:100 heparin was added. Activator was added to this solution so that there was a final concentration of 1:1000.

**Serum.** Blood was collected and allowed to clot at room temperature. After separation, the serum was stored either at -20°C or at 4°C. The day before testing, 0.25 ml of sera were placed in small tubes and inactivated in a water bath at 60°C for 20 minutes. The sera were then stored at 4°C for 60 minutes and again inactivated at 60°C for 20 minutes. To each serum was added 6 ml of buffered saline solution and the solution was stored at 4°C overnight.

**Standardization Procedure.** A preliminary standardization of the coagulase preparation is

performed by making serial two-fold dilutions in 1 ml volumes, using tryptose broth as a diluent. To each tube is added 1 ml of the fibrinogen solution containing 1:3500 activator. The rack containing the tubes is shaken, and placed in the 37°C water bath for 3 hours. The preliminary titer is then read as the highest dilution of coagulase which results in the formation of a visible clot.

The final titration employs the proper dilution of coagulase in tryptose broth as indicated by the preliminary titration. The coagulase is added to a duplicate series of 6 tubes in decrements of 0.1 ml. Tryptose broth is added to bring the volume up to 1 ml.

One ml of the fibrinogen standard is added, and incubation carried out as above. A unit of coagulase is then defined as the smallest amount which results in the slightest visible shred of fibrin after 3 hours' incubation. Reading of the test is facilitated by the use of a hand lens.

**Coagulase-inhibition Test.** Previous studies<sup>6,7</sup> have indicated that the union of coagulase and inhibitor is practically complete after 90 minutes' incubation at 37°C. The procedure employed for the titration of anticoagulase includes a preliminary and a fine titration as follows: to a series of 10 x 125 mm tubes containing 1 ml of various dilutions of serum previously inactivated as described above, is added 0.5 ml of coagulase containing 1 unit. After shaking, the mixtures are incubated at 37°C for 90 minutes and 0.5 ml of fibrinogen solution containing 1:1000 activator and heparin is added with an automatic pipette. The tubes are placed again in a water bath at 37°C for 3 hours. The titer of anticoagulase is read as the greatest dilution (initial) of serum which completely inhibits the formation of any visible shred.

In the preliminary titration the initial dilutions of serum employed are 1:25, 1:125, and 1:625. The final titer is then determined using 4 or 5 tubes of the proper dilutions as indicated in Table I.

**Accuracy.** To establish the accuracy of the

<sup>†</sup> Armour and Co.

<sup>‡</sup> The authors wish to thank Armour and Co., Chicago, Ill., for supplying this product.

<sup>6</sup> Lominski, I., and Roberts, A. B. S., *J. Path. and Bact.*, 1946, **58**, 187.

<sup>7</sup> Remmelkamp, C. H., unpublished observations.



In the digested smears the cytoplasm of the 12 basophiles studied was almost transparent and without color. It is therefore concluded that the material in the basophilic leucocytes which stained red in leucofuchsin after periodic acid is glycogen. Furthermore, since

glycogen can be demonstrated in the human basophilic leucocytes, they do "differ from mast cells which contain periodic acid-Schiff positive material which is insoluble in saliva." (Wislocki, Rheingold and Dempsey<sup>1</sup>).

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## A Quantitative Method for Measuring Staphylococcal Anticoagulase.\* (17382)

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The study of infections produced by *Staphylococcus aureus* in man or in experimental animals has been hampered by the lack of a suitable serological test. Since staphylococcal coagulase will stimulate the production of an inhibitor substance, termed anticoagulase,<sup>1,2</sup> it has been possible to devise a serological test that could be used in the study of infections caused by this organism.

The mechanism whereby staphylococcal coagulase clots plasma has been partially clarified by the recent work of Smith and Hale,<sup>3</sup> Kaplan and Spink,<sup>4</sup> and Tager.<sup>5</sup> Most strains of *S. aureus* produce an extracellular substance, coagulase, which, added to plasma, results in the formation of a clot. Coagulase does not clot fibrinogen directly, for it has been found that a second factor, termed acti-

vator<sup>3</sup> or reacting factor,<sup>5</sup> is also required. This substance is found in the blood of man and of certain animal species. Although it is not entirely clear at the moment whether coagulase, activator, or a product of these two substances is responsible for the clotting of fibrinogen, antibodies against coagulase develop following immunization with cell-free coagulase. By employing measured amounts of coagulase, fibrinogen and activator, it has been possible to develop a quantitative method for the measurement of coagulase inhibitor, anticoagulase.

*Materials and methods. Buffered Saline.* A solution of 0.01 M phosphate in 0.85% sodium chloride at a pH of 7.4 was prepared for dilution of all sera and for preparation of fibrinogen, activator and heparin solutions.

*Activator.* The source of activator was 300 ml of human serum from one donor. This standard serum was stored in glass containers containing 50-75 ml at 4°C.

Two strengths of activator were employed. For standardization of coagulase, a final dilution of 1:3500 of the serum in a solution of fibrinogen was employed. In the coagulase inhibition test a dilution of standard serum of 1:1000 was used.

*Heparin.* A solution of heparin containing 100 mg per 10 ml of buffered saline solution was prepared and stored at 4°C until used. A 1:100 dilution of this was added to the fibrinogen-activator mixture.

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<sup>1</sup> Rammelkamp, C. H., *Am. J. Med.*, 1948, 4, 782.

<sup>2</sup> Tager, M., and Hales, H. B., *J. Immunol.*, 1948, 60, 475.

<sup>3</sup> Smith, W., and Hale, J. H., *Brit. J. Exp. Path.*, 1944, 25, 101.

<sup>4</sup> Kaplan, M. H., and Spink, W. W., *Blood*, 1948, 3, 573.

<sup>5</sup> Tager, M., *Gale J. Biol. and Med.*, 1948, 20, 269.

TABLE II.  
Repeated Titrations of Anticoagulase of Control Sera.

Serum	Anticoagulase titer									
	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
1	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
2	<25	<25	<25	<25	<25	<25	<25	<25	<25	<25
3	278	417	278	278	278	179	179	417	417	278
4	278	278	278	278	278	179	278	417	417	278
A	179	125	179	125	179	—	125	—	—	—
B	900	900	900	900	900	1400	900	900	900	900
C	278	278	417	278	417	417	278	278	—	—
D	417	278	417	278	417	417	417	—	—	—

Anticoagulase titers of sera 1, 2, 3, and 4 were determined on different days between 7/15/48 and 8/2/48.

Sera A, B, C, and D were used as controls between 8/26/48 and 1/29/49.

sera be employed in the antibody titration within 24 hours since within 48 hours small amounts of activator may again be detected.

The system used for measuring inhibitor to coagulase employs 0.5 ml of fibrinogen containing 1:1000 activator, whereas in the titration of coagulase, 1.0 ml of fibrinogen containing a dilution of 1:3500 activator is used. This procedure of increasing the activator concentration in the antibody titrations was employed so that technical variations in the amounts of coagulase or activator added, or residual amounts of activator remaining in the serum being tested, would not alter the end-point appreciably. The present test, as compared to that devised by Lominski and Roberts,<sup>6</sup> controls the concentration of activator in the fibrinogen and therefore more reproducible results should be obtained.

The stability of the various reagents used in this test is not definitely known. The fibrinogen solutions should be employed within

a few hours of preparation. When stored at  $-20^{\circ}$  or  $4^{\circ}\text{C}$  in sealed ampules, coagulase retains most of its activity over a period of a year or more, but repeated standardizations are best performed at monthly intervals. Tryptose broth is added as a diluent to the coagulase because it appears to stabilize its action so that results are reproducible. Activator, when stored in sealed ampules at  $-20^{\circ}\text{C}$  or  $-4^{\circ}\text{C}$  or at  $4^{\circ}\text{C}$  in rubber stoppered flasks for over a year, appeared to have lost little activity. When stored at room temperature, however, a loss of activity was observed.

**Summary.** A satisfactory serological test for the titration of anticoagulase in human and animal sera has been devised by controlling the various factors which enter into the coagulase-anticoagulase reaction.

The technical assistance of Miss Margaret Hezebicks is gratefully acknowledged.

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## Effect of Dibuline\* on Nocturnal Gastric Secretion in Man. (17383)

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It has been shown<sup>1-5</sup> that dibuline (dibutyl urethane of dimethyl ethyl-B-

\* Supplied by Dr. M. I. Grossman, University of Illinois, Chicago, Ill.

<sup>1</sup> Swan, K. C., and White, N. G., *J. Pharm. and Exp. Therap.*, 1944, 80, 285.

hydroxyethyl ammonium sulfate, Merck) has a qualitative effect similar to that of atropine.

<sup>2</sup> Featherstone, R. M., and White, N. G., *J. Pharm. and Exp. Therap.*, 1945, 81, 165.

<sup>3</sup> Peterson, C. G., and Peterson, D. R., *J. Pharm. and Exp. Therap.*, 1945, 81, 256.

TABLE I.  
Method for Preparing Preliminary and Fine Titrations of Sera for Anticoagulase Titrations.

Preliminary dilution of serum	Serum, ml	Buffered saline, ml	Initial dilution
1/25	1.0	0	25
0.25 ml of serum	0.7	0.3	36
+	0.45	0.55	56
6.0 ml of buffered saline	0.3	0.7	83
1/125	1.0	0	125
1.0 ml of 1/25 serum	0.7	0.3	179
+	0.45	0.55	278
4.0 ml of buffered saline	0.3	0.7	417
1/625	1.0	0	625
0.5 ml of 1/25 serum	0.7	0.3	900
+	0.45	0.55	1400
12.0 ml of buffered saline	0.3	0.7	2100

test 4 control sera are included in all titrations of anticoagulase. The results of repeated tests on 2 sets of control sera are shown in Table II. Titers of sera run on different days varied only 2 dilution increments. It would appear that acute and convalescent sera exhibiting a difference in titer of 2 or more dilution increments, as determined on a single titration, represent a true difference in inhibitory substance. Occasionally it may be desirable to determine the amount of antibody in dilutions less than 1:25; in such cases the reproducibility of the titers is somewhat less accurate.

**Discussion.** The serological test described in the present report has been used for the past 2 years and numerous sera have been examined. The technic is simple; 100 or more sera may be titrated in one day. The test has the advantage that the various reacting substances are carefully controlled. A fixed amount of coagulase is allowed to react with dilutions of the serum to be tested, following which known amounts of fibrinogen and activator are added. The end-points are reproducible and easily read.

Living cultures of *S. aureus* have usually been employed in attempts to demonstrate an antibody to coagulase. Such a procedure precludes a quantitative measurement of the inhibitor since variable amounts of coagulase are produced during incubation of the plasma-coagulase mixtures. Cell-free coagulase may be obtained by heating or filtering cultures of *S. aureus*<sup>8</sup> or by the addition of bacterio-

static agents.<sup>9</sup> Variable results have been obtained as regards the resistance of coagulase to heat and filtration.<sup>8</sup> Acid cultures yield no coagulase upon heating, and alkaline cultures yield little or no coagulase on filtration. Lominski and Roberts<sup>6</sup> employed broth containing 10% plasma and found that coagulase would then pass through a filter. Originally we employed plasma in the culture medium, but later it was found that filtrates from such cultures clotted purified fibrinogen directly, indicating that a significant and variable amount of activator from the plasma remained in the culture filtrate. Since the measurement of coagulase is dependent upon the interaction of activator and coagulase, plasma cannot be used in the culture medium. For this reason bovine albumin was employed, and it was determined that coagulase became filterable in the presence of albumin.

Activator was obtained from one source: human serum from one donor known to be free of a coagulase inhibitor. Because this serum was capable of clotting plasma or fibrinogen solutions directly, heparin was added to the system. Experience has shown that most animal sera and an occasional human serum will clot fibrinogen directly. In the titration of antibody, sera to be tested are inactivated in order to remove the activator or reacting substance. It is important that inactivated

<sup>8</sup> Lominski, I., and Milne, J. A., *J. Path. and Bact.*, 1947, 50, 516.

<sup>9</sup> Tager, M., and Hales, H. B., *Yale J. Biol. and Med.*, 1947, 20, 41.

TABLE II.  
Effect of Dibuline (10 mg I.M. at 9:30 p.m., 1:30 and 5:30 a.m.) on 12-Hour Nocturnal Gastric Secretion in Man.

Case	Control			During Dibuline			% change		
	Vol. (cc)	Free HCl (Cl units)	Free HCl (mg)	Vol. (cc)	Free HCl (Cl units)	Free HCl (mg)	Vol.	Free HCl (conc.)	Total output HCl
1	435	75	1191	744	54	1456	+71	-28	+
2	998	89	3200	1456	79	4170	+46	—	+30
3	401	49	719	506	34	624	+26	-30	—
4	1188	42	1833	1047	69	2616	—	+64	+43
5	553	67	1352	671	76	1851	+	+	+37
6	922	59	1988	1103	65	2593	+	+	+30
7	583	60	1282	671	72	1761	+	+	+37
8	1188	90	3892	1074	94	3690	—	+	—
9	1026	83	3103	1207	78	3416	+	—	+
10	598	56	1211	616	35	790	+	-38	-35
11	740	58	1556	622	39	885	—	-33	-43
12	1008	33	1208	757	14	387	-25	-58	-68
13	1232	63	2802	726	33	878	-41	-48	-69
14	1098	48	1908	591	33	491	-55	-31	-74
15	572	42	875	214	68	526	-63	+64	-40

mg) seems comparable to 1.0 to 2.0 mg of atropine. The decrease in gastric secretion, when it occurs, persists usually for 2 to 3 hours only. The repeated administration of the drug 4 hours after the initial dose is no more effective than the initial injection.

Dibuline is tolerated better than atropine. The parenteral injection of the latter in 1.0 mg doses every 4 hours, very frequently produces marked dryness of the mouth, blurring of vision, and tachycardia. Repeated injec-

tions of 10 mg of dibuline every 4 hours very seldom produce side effects; when they do occur, however, the symptoms are mild and of brief duration.

*Conclusions.* The effect of dibuline on gastric secretion is variable, transitory, and unpredictable. Further trial of the drug in the treatment of peptic ulcer seems to us unwarranted.

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## Possible Sources of the Androgenic Factor in Cow Manure. (17384)

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The bacterial flora of the stomach of a ruminant is known to synthesize a number of compounds: proteins from urea;<sup>1</sup> vitamin B complexes;<sup>2,3</sup> etc. It was felt desirable to determine changes in 17-ketosteroids on incubated feces.

<sup>1</sup> Smith, J. A. B., and Baker, F., *J. Biochem.*, 1944, 38, 496.

<sup>2</sup> Hunt, C. H., Burroughs, L. W., Bethke, R. M., Schalk, A. F., and Gerlaugh, Paul, *J. Nutrition*, 1943, 25, 207.

<sup>3</sup> Burkholder, Paul R., and McVeigh, Ida, *Proc. Nat. Acad. Sci. U. S.*, 1942, 28, 285.

Turner<sup>4</sup> finds an androgenic factor in fecal material of pregnant cattle and goats. Longwell and Gassner<sup>5</sup> report increased size of comb when chicks are fed dried fecal material as part of their diet. They have made some attempt at characterization of the compound but have not reached a definite conclusion as to its chemical nature.

It seems desirable to investigate two pos-

<sup>4</sup> Turner, C. W., *J. Dairy Science*, 1947, 30, 1.

<sup>5</sup> Longwell, Bernard B., and Gassner, F. N., *Fed. Proc.*, 1947, 6, 272.

It possesses both a smooth muscle-inhibiting and an antiacetylcholine action. Marquardt, Case, Cummins, and Grossman<sup>6</sup> have reported a temporary decrease in the gastric secretory rate in dogs and humans given 10 mg subcutaneously; the effect was of short duration, the secretion returning to control levels within an hour. The purpose of this paper is to summarize a study of the effect of dibuline on the nocturnal gastric secretion of 14 patients with duodenal ulcer and one with functional bowel distress.

*Method of study.* The group consisted of 14 males and one female (14 with duodenal ulcer and one with functional bowel distress).

I. *The effect of a single injection of dibuline* was determined in 45 studies in fasting individuals. After a control period of 6 hours from 2:30 to 8:30 p.m., dibuline was given intramuscularly in a dose of 10 mg and its effect noted in the subsequent 4 hours. The gastric contents were aspirated continuously throughout the entire period of observation, the collection bottles being replaced at hourly intervals. The volume, concentration and total output of free hydrochloric acid were measured for each hour. A depressant effect on gastric secretion was considered significant if the reduction was greater than 25% and it was sustained for as long as 2 hours.

II. *Data on the total nocturnal gastric secretion* were obtained in a series of 15 experiments. The conditions of study were identical in all. All studies were made between 9:30 p.m. and 9:30 a.m. The gastric contents were aspirated continuously, the containers being replaced at hourly intervals. The volume, concentration of free hydrochloric acid, and total output of free hydrochloric of each hourly sample were determined by the usual method. Control hourly values for the 12-hour nocturnal gastric secretion were obtained for one night prior to the use of dibuline. During the second night, 10 mg of

TABLE I.  
Effect of 10 mg Dibuline I.M. on Gastric Secretion in Man.

	Vol., %	Conc. free HCl, %	Free HCl mg. %
Increase > 25%	31	26	33
No significant change	43	34	27
Decrease > 25%	26	40	40

dibuline in 1.0 cc of water were given intramuscularly at 9:30 p.m., 1:30 a.m. and 4:30 a.m. The patients did not experience any pain at the site of the injection. No effect was considered significant unless a change of 25% or more was obtained.

*Results. Effect of a single injection of dibuline* (Table I). The hourly volume was higher than the control values in 31% of the studies and did not change in 43%. A decrease of 25% or more from the control values was noted in 26% of the studies. Both the concentration and the output of free hydrochloric acid were lower in 40% of the studies. On the other hand, values greater than the control occurred during the administration of dibuline in 26 and 33%, respectively.

*Effect on the 12-hour nocturnal gastric secretion.* (Table II). In 3 individuals, the volume exceeded the control values. It was not affected significantly in 8 studies; lower volume was noted in 4 studies. In 3 of the latter, the concentration and output of free hydrochloric acid were diminished simultaneously. In 4 additional patients the concentration of free hydrochloric acid diminished although the volume of gastric secretion was unaffected. The total output of free hydrochloric acid (mg) was unchanged in 4 studies, considerably higher than the control values in 5, and lower in 6.

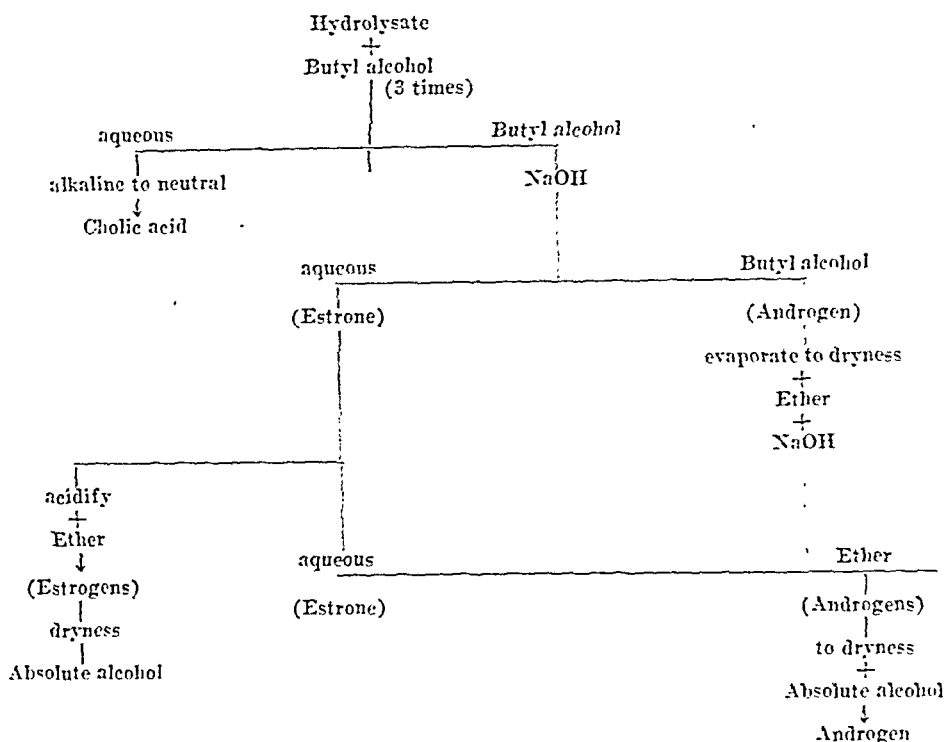
*Discussion.* The present results indicate that the effect of dibuline on gastric secretion in man is variable and similar to that obtained with atropine sulfate.<sup>7</sup> Both increases and decreases in secretion, as compared with the control period, were observed. The decreases particularly seem to be of sufficient magnitude to be of some significance. The recommended dose of dibuline (10

<sup>4</sup> Peterson, C. G., and Peterson, D. R., *Gastroenterology*, 1945, 5, 169.

<sup>5</sup> Cummins, G. M., Marquardt, G. H., and Grossman, M. I., *Gastroenterology*, 1947, 8, 205.

<sup>6</sup> Marquardt, G. H., Case, J. T., Cummins, G. M., and Grossman, M. I., *Am. J. Med. Sci.*, 1948, 216, 203.

<sup>7</sup> Levin, E., Kirsner, J. B., and Palmer, W. L., to be published.



and after the removal of the chromogens are shown below at 508  $m\mu$  for the original sample (No. 1) and the 2 which were incubated for one and 2 weeks (Nos. 2 and 3, respectively).

The K-value obtained is a comparative measure of the quantity of the 17-ketosteroids present. Since there was no increase in the K-value, it is conclusive that no increase of 17-ketosteroids was experienced on incubation and that therefore no increase of androgens was evident upon incubation as carried out. Since there was no increase in 17-ketosteroids, no biological androgenic assay was considered necessary to prove that the bacterial flora of the feces was not responsible for the increased androgenic content of pregnant cow manure.

TABLE I.

Sample	Col. I Before	Col. II After
1	1.524	1.533
2	1.164	no peak
3	1.255	1.322

*Results and conclusions.* 1. The Zimmermann test showed that there was no significant difference in the amount of 17-ketosteroids before and after incubation periods up to 2 weeks. Since the material tested in column II has been extracted thoroughly with 2N NaOH, little estrogenic substance can be present. Hence the figures obtained are believed to indicate androgens.

2. It is therefore believed that the bacterial flora of the ruminant stomach is not concerned with the increased androgenic content of the feces of a pregnant cow. If any changes are obtained, it appears that there is a loss of androgens during incubation, or a loss by the second treatment.

3. The bile from pregnant cows showed a demonstrable amount of an androgenic substance.

The author wishes to acknowledge the invaluable technical assistance rendered by Miss Marion Bucklin for this paper.

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sible sources of the androgens found in the feces of pregnant cattle and to study the properties of the material extracted: first, its production by bacterial flora upon incubation of fecal material; and secondly, its presence in demonstrable amounts in the bile of pregnant cows. Immature rats, ovariectomized female rats and single comb White Leghorn baby chicks, both sexes, served as experimental subjects.

*Experimental. Collection and disposition of samples of feces.* The cow manure, free of urine, was collected within 2 hours after dropping from a pregnant cow of 7½ months duration. The sample was thoroughly mixed and divided into 3 portions which were placed in sterilized fermentation jars. Sample No. 1 weighed 3333 g; No. 2, 3379 g; No. 3, 3751 g. Sample No. 1 was immediately covered with absolute alcohol in such amounts that with the water present, the mixture was about 70% alcohol. The untreated samples No. 2 and No. 3 were placed in an incubator at 37.5°C, the former remaining for one week, and the latter for 2 weeks.

*Extraction of feces.* The feces, which was placed in absolute alcohol as indicated above, was extracted in the cold 3 times with agitation, using calculated amount of 95% alcohol to give about 70% concentration. The combined extracts were allowed to stand overnight, centrifuged and decanted. This solution was evaporated to dryness under reduced pressure to a small volume. It was then boiled with norite and filtered through supersal to remove colored matter. The alcohol solution was evaporated to dryness under reduced pressure and this procedure repeated until all the water was removed. The residue was taken up in alcohol so that 1 ml contained the equivalent of 15 g of fresh manure. After their respective periods of incubation, samples No. 2 and No. 3 were treated in a like manner. All were very dark in color, which boiling with norite and filtering through supersal did not remove.

Such high dilutions had to be made in order to be able to apply the Zimmermann test, that the following further attempt was made to remove the chromogenic material. An aliquot of each sample was evaporated to dryness

under diminished pressure, taken up in water and extracted 3 times with carbon tetrachloride. These were then washed twice with 2N NaOH, and once with 10 cc of Lycopon (1N NaOH with 10 g of sodium hydrosulfite). The  $\text{CCl}_4$  was evaporated to dryness under diminished pressure and the residue dissolved in alcohol. Samples No. 1 and No. 3 still were a dark brown color and No. 2 was a bright yellow color. Later tests with the Zimmermann test indicate that for some reason No. 2 gave no definite absorption band at 508 m $\mu$ .

Following the alcoholic extraction of the feces, some of the extract from sample No. 1 was then evaporated to dryness and extracted 3 times with ether. The ether was evaporated to dryness under diminished pressure, and the residue dissolved in absolute alcohol. This will be referred to as sample No. 4.

*Extraction of bile.* Bile was obtained from pregnant cattle of 3-4 months duration. To it was added one-fourth its volume of concentrated hydrochloric acid, and the mixture boiled for 15 minutes. The hydrolysate was then treated as shown on following page.

*Test for androgenic factor using baby chicks; application of extract of feces.* Two-day-old single comb White Leghorn chicks of both sexes were used in the test. The extract, equivalent to 3 g fresh manure, was applied to the comb each day for about 4 weeks. This was done only on the fresh extract and not on incubated fecal material in order to demonstrate the presence of androgens in the sample under consideration. Increased comb growth was noted in both male and female chicks, but it is not of mathematical significance.

*Application of extract of bile.* The extract of bile was applied topically exactly as described for application of fecal extract. An equivalent of 3 ml of fresh bile was applied daily on each chick's comb. An increase in comb growth of both male and female chicks was demonstrable.

*Spectrophotometric studies.* Samples before and after attempt to remove chromogens were equilibrated and tested for 17-ketosteroids by the Zimmermann reaction. K-values (where  $K = \log I_0/I$ ) for these before

TABLE I.  
Effect of a Single Injection of Testosterone Propionate on Alkaline Phosphatase Content of Mouse Thyroid Gland.

Testosterone propionate dosage, mg	Mice fed fox chow only	Mice fed fox chow plus 0.25% thiouracil	Mice fed fox chow plus 0.10% thyroglobulin
0	+	0	+
.05	+	0	+
.10	+	0	+
.20	+	0	+
.50	++	0	+
2.50	+++		

0 = An overall negative phosphatase content.

+ = Nuclear and slight cytoplasmic content.

++ = Nuclear and moderate cytoplasmic content.

+++ = Fairly heavy content in the nuclei and cytoplasm of the acinar cells; slight content in the nuclei of the interfollicular cells.

at 22 days of age with autopsy at 32 and 39 days of age. The androgen, when administered, was injected on the 32nd day of age and permitted to act over 7 days thus a preliminary drug feeding period of 10 days preceded injection and in all cases drug feeding was continued after hormone administration.

Mice were killed by cervical dislocation. The right thyroid fixed in cold 80% alcohol and the left thyroid in Bouin's fluid. Alkaline glycerophosphatase was checked on sections cut at 7  $\mu$  and prepared by the Gomori technique<sup>11</sup> with the addition of a 2%  $MgSO_4$  solution to the incubation mixture to enhance the stain. Incubation was carried out at 37° for 7 hours at pH 9.2-9.4. The Bouin's fixed material was sectioned at 10  $\mu$  and stained with hematoxylin and eosin.

The thyroid gland of immature female mice has alkaline glycerophosphatase essentially concentrated in the nuclei of the follicular cells with a tinge appearing in the cytoplasm. This condition was not altered by testosterone propionate until a dosage of 0.5 mg was reached when, however, the enzyme concentration exhibited an increase in the cytoplasm in all 8 mice studied (Table I). This proved a critical level, but an even more striking effect on the cytoplasmic phosphatase could be seen with a 2.5 mg dosage. Routine sections stained with hematoxylin and eosin failed to reveal an androgen effect.

Thiouracil feeding resulted in the anti-

pated hypertrophy of the thyroid epithelium and loss of colloid. Alkaline phosphatase was essentially lacking as the enzyme was not present in the cytoplasm and was reduced or absent in the nucleus of the follicular cells. Only the endothelial lining of the blood vessels contained alkaline phosphatase to any degree. Administration of testosterone propionate failed to alter this essentially negative enzyme picture even at the 0.5 mg dose which was effective in the normal mouse (Table I). The general histology also was not altered.

Following thyroglobulin feeding the thyroid histology was that of an atrophic gland as the follicular epithelium was now low cuboidal or squamous. Alkaline phosphatase was definitely present in the nucleus and the cytoplasmic staining concentrated so that the follicular epithelium frequently appeared as a line of alkaline phosphatase. Essentially the same histology has been described for the rat thyroid following hypophysectomy.<sup>12</sup> Testosterone propionate in the dosages used failed to alter the alkaline phosphatase histology and provided no evidence of an effect in our routine preparations (Table I).

Increased activity of the thyroid gland is correlated with a loss in alkaline phosphatase whether induced by stress or thiouracil feeding. Furthermore, pituitary activation of the thyroid or disuse atrophy due to thyroid administration plays a primary role in the control of enzyme concentration since testosterone propionate can not override their effects. Nevertheless, in a normal female mouse, androgen can increase the alkaline

<sup>11</sup> Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, 1939, 12, 23.



# Alkaline Phosphatase in the Mouse Thyroid Following Testosterone Propionate, Thiouracil and Thyroglobulin.\* (17385)

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The BMR of patients is increased by testosterone propionate and suggests that this androgen might influence the thyroid gland.<sup>1</sup> In animals, testosterone propionate has been reported to increase mouse thyroid weight<sup>2</sup> and to increase mitotic figures in rat thyroids.<sup>3</sup> It should be noted, however, that other investigators have failed to find an effect of testosterone on thyroid weight in mice<sup>4</sup> and rats<sup>5</sup> as well as in rats fed thiouracil.<sup>5,6</sup> Since weight was the essential criterion of effect and this factor is variable, it seemed more plausible to reinvestigate this problem on a histochemical basis and the alkaline glycerophosphatase technic was chosen.<sup>7</sup> This enzyme was not found in the rat thyroid in initial studies<sup>7-9</sup> but Dempsey and Singer<sup>10</sup> obtained positive results with longer incubation times. Alkaline phosphatase of the mouse thyroid has not been described but our preliminary studies revealed its presence making possible the androgen study in this species.

In addition to the effect of testosterone propionate on the normal thyroid it was of

interest to determine whether the androgen would influence a thyroid gland altered by thiouracil or thyroglobulin feeding. Changes in physiological state may alter the alkaline phosphatase concentration. For example, stress induced by cold reduced enzyme concentrations<sup>11</sup> but no definite conclusions were drawn from thyroids of 2 rats fed thiouracil.<sup>10</sup> Kroon<sup>12</sup> observed an increased alkaline phosphatase in the distended thyroid blood vessels following thiouracil feeding to rats. Hypophysectomy did not effectively alter the alkaline phosphatase of the rat thyroid follicle.<sup>13</sup> The influence of thiouracil or thyroglobulin feeding alone on the mouse thyroid is presented.

Twenty-two day old Swiss albino female mice were used and maintained on Purina Fox Chow. Groups of 7-11 animals were given testosterone propionate (Perandren, Ciba†) in a single injection of 0.05 mg, 0.10 mg, 0.20 mg, or 0.50 mg when 32 days old. One group of 5 animals received a single injection of 2.5 mg of the androgen. The mice were sacrificed 7 days later. Control mice were autopsied at 32 and 39 days of age.

To study the influence of hypo- and hyperthyroidism alone and with testosterone propionate, 0.25% thiouracil or 0.1% thyroglobulin (Proloid) was incorporated in the Fox Chow diet. The drug feeding was started

\* This investigation was done under contract with the Office of Naval Research, Navy Department.

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† Testosterone propionate (Perandren, Ciba) was supplied by Dr. E. Oppenheimer, Ciba Pharmaceutical Products, Summit, N. J., Thiouracil (Deravet) was supplied by Dr. Mark Welsh, Lederle Laboratories, Pearl River, N. Y., and Thyroglobulin (Proloid) was provided by Dr. Robert Kroe, The Maltine Company, Morris Plains, N. J.

TABLE II.

Specimen	Lysozyme titer in units/g or cc
1. Human fetal tissue (13 wk)	
a. whole intestine	39.8
b. thigh muscles	33.4
c. lung	13.3
d. iliac bone	25.0
e. skin	19
f. placenta	100
g. " "	100
2. Dog amniotic fluid	0
3. Two full term placentas (human)	22.9 61.6
4. Endometrium (human)	
a. 14th day of cycle	8.1
b. 26th day of cycle	14.8
c. 3rd day menstrual discharge	27.8
d. bloody discharge following 4 months miscarriage	18.5
5. Nephrotic urine (normal urine—less than 1)	6
6. Pus	
a. from acute staphylococcal abscess over elbow joint	237
b. from chronic <i>Clostridium welchii</i> peritoneal abscess	80
c. from chronically infected pilonidal abscess (anerobic strep.)	280
7. Mass cultures of intestinal flora in two patients with chronic ulcerative colitis	0 ; 0
8. Stool from cases of acute gastroenteritis due to	
a. <i>Salmonella typhimurium</i>	6.3
b. Paratyphoid B	11.5
9. Paratyphoid B culture	0
10. Acute non-specific infantile diarrhea-stool	
a. 1st day of disease	125
b. 7th day of disease	5
11. Dog gastric juice	
a. under anesthesia	2.3; 1.6; 5.5
b. conscious (acidity 0/4, 0/28)	5.3; 21.3
12. Enterocystoma fluid (small bowel mesentery)	125

maining in the gut over time. It is considered that this explains the two-way variation in these 3 cases.

A comparison of these values to those previously reported in chronic ulcerative colitis, normal subjects, and normal subjects with purging shows the definite elevation of lysozyme concentration in regional enteritis. This confirms the results in the 6 cases already reported.

In addition, 3 samples of mucosa from resected segments of involved ileum were assayed. They contained 12, 66.6 and 36.2 units/g of mucosa respectively.

These data confirm the previously published impression that regional enteritis is a disease characterized by high lysozyme concentrations in the stool and in the mucosa. The mucosal assays suggest that the pathogenesis of this entity is identical with that of chronic ulcerative colitis, and that the pathological differences between them are caused by the variable response of any one tissue to injury. The cause of the localization of increased mucosal lysozyme in any one gut segment is not known.

The lysozyme titres associated with the normal gastrointestinal tract, peptic ulcer,

phosphatase concentration suggesting the use of histochemical technics to evaluate steroid hormone actions on the thyroid and at the same time emphasizing the importance of the physiological state of the end organ and its effect on anticipated results.

*Summary.* Testosterone propionate definitely increased the cytoplasmic alkaline phosphatase in the follicular cells of the mouse thyroid when dosages of 0.5 mg or more were

used. Thiouracil (0.25%) induced marked hyperplasia in 17 days and virtually eliminated the alkaline phosphatase in the follicular epithelium whereas thyroglobulin caused a follicular cell atrophy without loss of the enzyme. Testosterone propionate did not influence the thyroid alkaline phosphatase in the presence of thiouracil or thyroglobulin.

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### Lysozyme Titres in Regional Enteritis, Miscellaneous Tissues, Microorganisms, and Excreta.\* (17386)

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(Introduced by Karl Meyer.)

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The apparent increase in the lysozyme content of the stools in regional enteritis has already been reported.<sup>1</sup> At that time the proposition that lysozyme is a local initiating agent in the pathogenesis of this disease (as well as in chronic ulcerative colitis) was advanced. The circumstantial evidence supporting this conclusion was reported there and in an accompanying article discussing the probable etiologic role of lysozyme in peptic ulcer.<sup>2</sup>

Only 6 cases of regional enteritis were reported at that time; it therefore appears desirable to supplement this evidence with that subsequently obtained.

The values in the additional cases studied appear in the table. All were regional ileitis with involvement of varying lengths of bowel. The disease process ceased at the ileo-cecal valve in every instance. The assays were done by the viscosimetric technic of Meyer.<sup>3</sup>

The results were markedly uniform except

for 2 low titres and one quite high one. The former were from constipated patients and the latter individual had marked diarrhea. It has already been noted that the rate of fecal expulsion influences the stool titre, particularly when the disease process does not extend to the descending colon. This depends upon the gradual inactivation of fecal lysozyme re-

TABLE I.

Case	Lysozyme concentration in units/g of stool (wet wt)	Lysozyme output per day in units
1	17.8	1,727
2	3.6	—
3	14.8	17,538
4	18.2	1,170
5	32.9	3,258
6	21.3	—
7	1.7	1,120
8	22.4	2,910
9	15.4	—
10	22.2	—
11	14.3	—
12	17.0	—
13	20.4	—
14	55.0	15,817
Means	19.8	6,220

\* Supported in part by the Research Grants Division of the U. S. Public Health Service.

<sup>1</sup> Meyer, K., Gelhorn, A., Prudden, J. F., Lehman, W. L., and Steinberg, A., *Am. J. Med.*, 1948, 5, 496.

<sup>2</sup> Meyer, K., Prudden, J. F., Lehman, W. L., and Steinberg, A., *Am. J. Med.*, 1948, 5, 482.

<sup>3</sup> Meyer, K., and Hahnel, E., *J. Biol. Chem.*, 1946, 163, 733.

Avg stool lysozyme values in normal individuals (1) 2.7 158

Avg stool lysozyme values in purged individuals (1) 1.6 1,064

Avg stool lysozyme values in chronic ulcerative colitis (1) 73.6 26,568

## Viral Agglutination of Saponin-Lysed Chicken Erythrocytes. (17387)

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The results of recent studies concerning the mechanism of hemagglutination by viruses have brought out certain facts pertaining to the presence, on or within the surface structure of agglutinable erythrocytes, of receptor-like substances which can be removed by appropriate procedures. It has been established,<sup>1</sup> for example, that treatment of human group O erythrocytes with a filtrate of a broth culture of *Vibrio comma* results in a suspension of cells which have lost their capacity to adsorb or be agglutinated by influenza virus. It appears that substances with such receptor-destroying properties produce no drastic alteration of the red cell architecture. This is evidenced by the fact that treatment of a suspension of chicken erythrocytes with influenza virus renders the cells receptor-free without producing any obvious change in the appearance of the suspension or in the morphology of the component cells. On the other hand, normal chicken erythrocytes which have been lysed, or partially lysed, by mechanical trauma, water, or dilute saponin retain the ability to adsorb<sup>2,3</sup> and be agglutinated<sup>4</sup> by influenza virus. A consideration of these facts raised the question as to whether a hemolytic agent such as saponin has no effect on the surface receptors or whether additional "internal" or "nuclear" receptors are exposed by the lytic process. The present paper provides information in support of the former possibility in that it is shown that chicken erythrocytes rendered inagglutinable by treatment with influenza virus are not agglutinated by the virus after lysis in dilute saponin. Experiments in this connection were prefaced by a quantitative study of the agglutinability of

saponin-lysed normal erythrocytes by the virus. Methods were developed for the preparation and use of suspensions of lysed cells in agglutination and inhibition titrations.

*Preparation of suspensions of lysed cells.* It was observed that if chicken erythrocytes were lysed with saponin by appropriate procedures, resulting suspensions were homogeneous, colorless, and opaque. Examination in a wet mount by ordinary or phase microscopy revealed the presence of particles which superficially resembled erythrocyte nuclei. The periphery, however, was not discrete and had a coarsely granular appearance. A considerable amount of amorphous extranuclear material, apparently stroma, was seen in heavily stained films.

Lysed cells can be used in agglutination (CCA) and -inhibition (CCAI) titrations, set up according to the Salk method,<sup>5</sup> only if the suspensions are prepared properly. It was found that almost every step in the procedure is critical. The ratio of number of cells to volume of saponin is of some importance, but of more importance are the concentration of saponin and the time the cells are allowed to remain in the saponin before centrifugation and washing. The lysing procedure that was adopted is as follows: one ml of adequately washed and packed chicken erythrocytes are added to 100 ml of 0.125% saponin. The cells are allowed to lyse for 6 minutes with constant shaking by hand. The suspensions are centrifuged at low speed (1800 R.P.M.), and are washed twice and resuspended in 0.9% NaCl.

Suspensions of lysed cells prepared in this manner were used in the early phases of the work. CCA and CCAI tests were not easy to read, however, in that the pellets and agglutination patterns were colorless. This situation was remedied by the addition of 2-3 ml of a 0.5% aqueous methyl green solu-

<sup>1</sup> Burnet, F. M., *Brit. J. Exp. Path.*, 1946, **27**, 228.

<sup>2</sup> Hirst, G. K., *J. Exp. Med.*, 1942, **76**, 195.

<sup>3</sup> Lajmanovich, S., and Mittleman, N., *Rev. Soc. Argentina Biol.*, 1946, **22**, 339.

<sup>4</sup> Carlisle, H. N., and Elrod, R. P., *Soc. Am. Bact. Proc. Meetings*, 1949, **2**, 92.

<sup>5</sup> Salk, M. E., *J. Immunol.*, 1944, **40**, 87.

chronic ulcerative colitis, body fluids and secretions, hyaline cartilage, and granulation tissue have previously been reported in detail.<sup>1-4</sup> It is the purpose here to record additional noteworthy assays.

The results are shown in Table II.

Many of these findings cannot be discussed because of lack of knowledge concerning the physiologic role of lysozyme. Some determinations, however, deserve comment because of their relation to inflammatory intestinal disease. For example, high stool lysozyme titres apparently are not a necessary corollary to acute specific gastrointestinal inflammation (see 8 in table). On the other hand, a previously reported<sup>1</sup> case of acute amebic dysentery had a stool titre of 38 units/g (upper limit of normal stool lysozyme concentration is about 9 units/g). Likewise, one case of acute non-specific diarrhea in an infant had a high stool titre which fell rapidly to normal over 7 days (see 10 in table). This case had no gross or occult blood in the stool.

The high lysozyme content of one of the 2 canine gastric juices obtained by Levine tube aspiration without anesthesia (? psychic stimulation) is of interest because a great many determinations on dog gastric juice, gastrointestinal mucosa, and stools in the absence of exciting or disturbing factors have been uniformly less than 6 units/g or cc.

The absence of any lysozyme in the mass cultures from chronic ulcerative colitis cases (see 7 in table) confirms the impression that intestinal lysozyme is not bacterial in origin.

*Summary.* The stools of 14 regional enteritis cases possessed a mean lysozyme content of

19.8 units per gram of stool (wet weight) and a mean daily output of 6,220 units. This is 7.34 times the mean lysozyme content of normal stools and 39.4 times the mean daily output in the stools of normal persons. These data, together with the experimental production by lysozyme of ulcerations in the canine alimentary tract,<sup>2</sup> indicate that lysozyme is an etiologic agent in the pathogenesis of regional enteritis.

A survey of various microorganisms, tissues, and excreta revealed generally high titres in fetal tissues and placenta, moderately high levels in endometrium with a suggestion of cyclic variation, and very large amounts in pus and in fluid from an enterocystoma. Low titres were found in the stools of two acute specific dysenteries and in canine gastric juice, with no measurable amounts in mass cultures from two chronic ulcerative colitis cases and one paratyphoid B culture. A case of acute infantile diarrhea exhibited a very high titre on the day of onset in the absence of organic pathology or specific infection, falling rapidly to normal with recovery.

It is felt that the generally high titres of fetal tissue suggest the possibility of an important role for lysozyme in the chemistry of actively growing normal tissue. The absence of lysozyme in the colitis mass stool cultures substantiates the conclusion that intestinal lysozyme is not bacterial in origin; and the moderately low levels in the specific dysentery stools indicate that acute colitis is not necessarily accompanied by a high stool lysozyme titre.

We acknowledge the generous advice of Dr. Karl Meyer and Dr. John S. Lockwood.

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<sup>4</sup> Prudden, J. F., Lane, N., and Meyer, K., to be published.

TABLE I.

Agglutinability of Normal and Receptor-Free Erythrocytes Before and After Saponin Lysis.

	Virus dilutions							Saline
	10	100	200	400	800	1600	3200	
Normal, lysed	+++*	+++	+++	+++	+++	+R	RR	PP
Receptor-free, lysed	PP†	PP	PP	PP	PP	PP	PP	PP
Normal, not lysed	+++	+++	+++	+++	+++	+++	PR	PP
Receptor-free, not lysed	PP	PP	PP	PP	PP	PP	PP	PP

\* Duplicate tubes.

+ Complete agglutination.

R—Ring, partial agglutination.

P—Pellet, no agglutination.

agglutinability of saponin-lysed cells by the virus is, in all probability, due to unaltered surface receptors and not to any substance associated with the nucleus. This interpretation is valid, however, only if it can be assumed that virus particles do not penetrate to

the interior of the erythrocytes during treatment with the virus. That such a process could occur seems unlikely in view of present knowledge concerning the interaction between red cell and virus.

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### The Tinctoral Demonstration of a Glycoprotein in Whipple's Disease. (17388)

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The etiology and pathogenesis of Whipple's Disease (lipodystrophy intestinalis), a clinical syndrome similar to sprue, has been a subject of speculation since its original description.<sup>1</sup> As its synonym implies, it is believed by most observers to represent an obscure disturbance of fat metabolism.

Black-Schaffer, Hendrix and Handler<sup>2</sup> reported a study of 4 cases which led them to the following conclusion: The disease, in contrast to sprue, may be readily recognized, anatomically, by non-lipid macrophagocytosis in the lamina propria of the small intestine and occasionally the proximal colon, lipogranulomatosis of the mesenteric lymph nodes, absence of significant evidence of chylous obstruction; and clinically, poor fat, glucose and probably protein absorption and the absence of macrocytic anemia.

The characteristic intestinal lesion is a crowding of the lamina propria by macrophages containing an isotropic refractile substance which Whipple found unstainable with osmic acid. This observation has been repeatedly overlooked in the literature, almost all authors assuming a lipid nature for this curious substance. The study of Black-Schaffer, Hendrix and Handler confirmed Whipple's observation. In 3 cases\* the phagocytosed material did not stain with Sudan IV, and 2† were likewise negative with Nile blue sulphate as well as osmic acid. Chemical analysis of the intestinal mucosa of two cases revealed no increase, over normal controls, of the lipid content.

The characteristic enlarged, cystic, fat-filled mesenteric lymph nodes (lipogranulomatosis) are so prominent that they have dominated the

<sup>1</sup> Whipple, G. H., *Johns Hopkins Hosp. Bull.*, 1907, 18, 382.

<sup>2</sup> Black-Schaffer, B., Hendrix, J. P., Handler, P., *Am. J. Path.*, 1948, 24, 677.

\* No suitable material was available from one case.

† No suitable material was available from 2 cases.

tion to the 100 ml of saponin used for lysis. In this manner, the erythrocytes were lysed and stained simultaneously. The use of methyl green stained suspensions definitely facilitated the reading of agglutination and inhibition titrations. Pellets were blue-green in color and the supernatant was colorless. The staining procedure did not affect the agglutinability of the particles. It appears that methyl green does not have the sensitizing effect in this reaction that it does in certain other agglutination systems.<sup>6</sup>

*Use of lysed cells in CCA and CCAI tests.* Suspensions of lysed erythrocytes were used to measure the CCA capacity of allantoic fluids containing the PR8 and Lee strains of influenza virus. Antisera against these agents prepared in chickens and rabbits were utilized in the CCAI titrations. All tests were set up according to the method of Salk.<sup>5</sup> When methyl green stained suspensions were used, pellets, rings and positive patterns were as discrete, and end-points were as definite and readable as in parallel control titrations in which normal erythrocytes were used. However, since the particles in the suspensions of lysed cells were smaller than erythrocytes, they sedimented more slowly, and a longer time was required for good pellet formation. All tests were read after storage overnight at approximately 4°C.

The end-points of agglutination titrations were governed by the concentrations of the suspension of lysed cells. As the concentration was decreased, the agglutination titer of virus preparations increased. Since this relationship between concentration and titer existed, suspensions of lysed cells could be adjusted with saline so that they yielded the same end-points as were obtained in parallel titrations of virus against normal erythrocytes. And, if such adjusted suspensions were used in inhibition titrations, end-points were the same as when erythrocytes were used. It was determined that suspensions of lysed cells which gave these similar titers contained 2.5 times as many particles per unit volume as did the 0.25% erythrocyte suspension used in the control tests. The signifi-

cance of this observation is being considered and will be discussed in a later communication.

*Experiments with receptor-free erythrocytes.* From the results obtained with normal erythrocytes it appeared that saponin had no effect on the receptors concerned in the agglutination of the cells by influenza virus. The possibility existed, however, that the surface receptors were destroyed and that additional receptors located within the cell were exposed by the lytic process. In view of this contingency, it was decided to investigate the agglutinability of suspensions of lysed cells prepared from chicken erythrocytes which had been rendered inagglutinable by treatment with virus. Receptor-free cells were prepared by the following method: One ml of adequately washed and packed erythrocytes was added to 9 ml of undiluted PR8 allantoic fluid (Salk titer, 2560). The virus-cell suspension was placed at 37°C for 6 hours, and was shaken gently every 30 minutes. The cells were then washed twice with 0.9% NaCl and packed to the original volume, one ml. The amount of hemolysis and change in color which occurred during the incubation period were negligible and did not exceed that observed in a similarly incubated 10% suspension of normal cells in saline. Receptor-free cells prepared in the above manner were not agglutinated by the virus. When used in Salk titrations, the pellets obtained in tubes containing virus were as well-formed as those in either the treated cell-saline or the normal cell-saline control tubes.

Virus-treated erythrocytes were lysed with saponin according to the method described previously. The resulting suspensions were indistinguishable grossly and microscopically from suspensions of lysed normal cells. They were not, however, agglutinable by influenza virus. Table I summarizes the results of one of three identical experiments in this connection.

*Comment and conclusions.* From these experiments it would seem that saponin has no effect on the receptors responsible for the agglutination of chicken erythrocytes by influenza virus. Internal receptors, if such exist, are not exposed during the lytic process. The

<sup>6</sup> Berger, F. M., *Brit. J. Exp. Path.*, 1943, 24, 252.

tive. On the other hand the mucicarmine stain<sup>5</sup> for mucus lends a faint tint to the phagocytosed substance. This, plus its insolubility in water—properties akin to those of the glycoprotein mucin which also stains scarlet to red with periodic acid—indicates that the polysaccharide is bound to a protein, thus constituting a glycoprotein.

The diminished fat, glucose, and possibly protein absorption by the small intestine in Whipple's disease indicates that its etiology resides in a disturbance of function of the intestinal epithelium. It is probably this defect which permits absorption of the unusual glycoprotein which may or may not be re-

lated to the mucin discharged into the enteric lumen by the intestine itself. At any rate, the presence of this readily demonstrated glycoprotein in the intestinal mucosa and mesenteric lymph nodes, indicates that Whipple's disease is more than an obscure defect in fat metabolism and is certainly not the result, as is commonly suggested, of a block of the mesenteric lymphatics. Thus the name lipodystrophy intestinalis, first proposed by Whipple and currently in use, would seem to be inappropriate. It would appear desirable to return to the eponym "Whipple's disease" until a name denoting the nature, rather than a complication, of the disease is forthcoming.

<sup>5</sup> Mallory, F. B., *Pathological Technic*, W. B. Saunders Co., Philadelphia, 1938.

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### Inhibitory Effect of Cow's Milk on Influenza Virus Hemagglutination.\* (17389)

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The development of theories on the mechanism of infection by influenza viruses has received considerable encouragement in the past few years from studies of the interaction between these viruses and inhibitors of virus hemagglutination found in various mammalian and avian fluids.<sup>1-7</sup> For some lines of

inquiry it is desirable to compare the properties of several fluids, as well as those of the inhibitors isolated from them. It is of interest, therefore, that cow's milk, a fluid readily available in considerable quantity, exerts an inhibitory effect on virus hemagglutination. Some aspects of this inhibition phenomenon are described in the present report.

\* This work was supported by the Commission on Influenza, Army Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D.C., and by a research grant from the National Cancer Institute, U. S. Public Health Service.

<sup>1</sup> Burnet, F. M., *Lancet*, 1948, 234, 7.

<sup>2</sup> Hirst, G. K., *J. Exp. Med.*, 1948, 87, 301, 315.

<sup>3</sup> Lanni, F., and Beard, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1948, 68, 442.

<sup>4</sup> Anderson, S. G., Burnet, F. M., Fazekas de St. Groth, S., McCrea, J. F., and Stone, J. D., *Austr. J. Exp. Biol. and Med. Sci.*, 1948, 26, 403.

<sup>5</sup> Svedmyr, A., *Brit. J. Exp. Path.*, 1948, 29, 295, 309.

<sup>6</sup> Hardy, P. H., Jr., and Horsfall, F. L., Jr., *J. Exp. Med.*, 1948, 88, 463.

<sup>7</sup> Francis, T., Jr., and Minuse, E., *Proc. Soc. Exp. Biol. and Med.*, 1948, 69, 291.

*Materials and methods.* Fresh raw milk was obtained from individual cows or as a pool from a commercial distributor and was skimmed by centrifugation in the cold room in the laboratory. The cream, which carried an insignificant part of the total inhibitory activity, was lifted off with a spatula and discarded. If necessary, the milk was recentrifuged to remove residual cream or sediment. Raw skim milk (RSM), less than one day old, was used for most of the experiments; if intended for use after one day, the milk was preserved with 1:5,000 Merthiolate (Lilly), which was found not to affect the inhibitory activity.



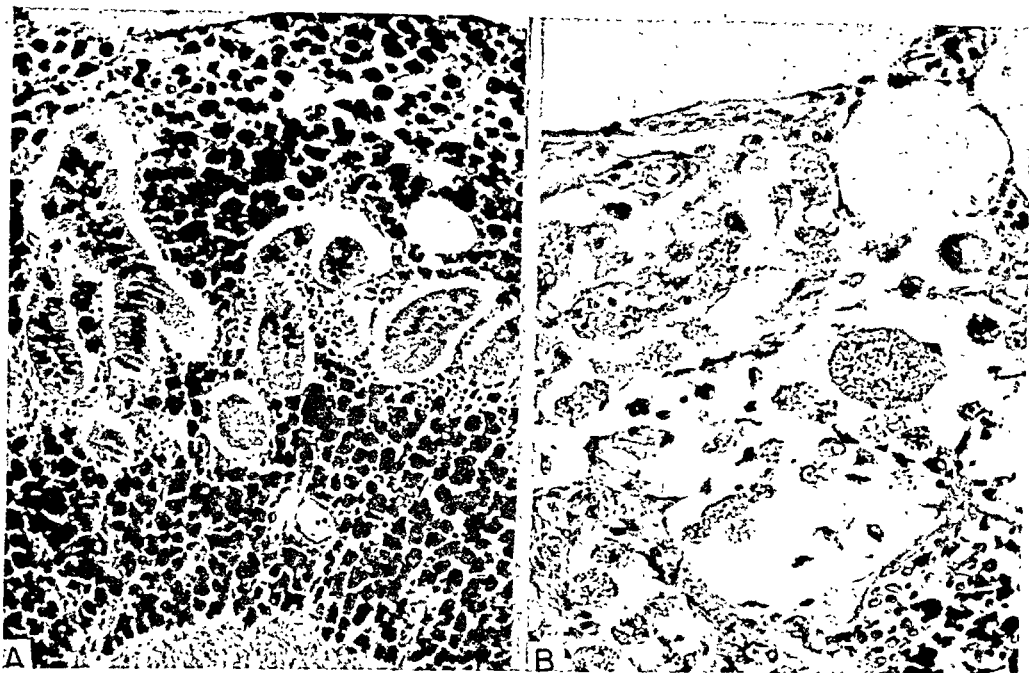


FIG. 1.

A. The mucosa of the small intestine in Whipple's disease occupied by macrophages filled with glycocalyx, stained by MacManus' modification of Schiff's periodic acid stain. The mucin of the "goblet cells" in the glands shows identical tinctorial properties.

B. Macrophages and giant cells in a mesenteric lymph node of Whipple's disease, revealing the presence of glycocalyx granules (in the photograph black) and lipid (colorless vacuoles). In the lower right corner are macrophages replete with glycocalyx and identical to those pictured in A.

approach to the problem.

Careful examination of the nodes revealed,<sup>2</sup> adjacent to sudanophilic macrophages, others containing sudanophobic substance similar in all respects to that described in the intestine. As a consequence of the pathologic anatomy, the histochemistry and the chemical analyses, a pathogenesis of the lesions was proposed.<sup>2</sup>

The present report is occasioned by the identification of the unknown phagotaxic substance as a glycocalyx. When sections of the intestines of 4 cases were treated with Schiff's periodic acid stain<sup>3,4</sup> the phagocytosed material in the mucosa stained deep scarlet (Fig. 1).

The lymph nodes of 3<sup>†</sup> of the cases con-

firmed the studies<sup>2</sup> of the fat-stained tissues. Many macrophages, in fact, surprisingly many, color a brilliant scarlet. Most of the giant cells surrounding the large masses of fat contain the same red substance (Fig. 2). Frozen sections treated with periodic acid and Sudan IV demonstrate lipid and polysaccharide side by side within the same macrophages. There is, however, no coalescing of the glycocalyx material to form amorphous masses as does the fat.

The periodic acid stain depends upon the oxidation of a 1-2 glycol bond in a polysaccharide forming polyaldehydes which thereupon take up the fuchsin of Fuelgin's reagent.

The polysaccharide containing material is insoluble in water, being present in apparently undiminished amounts after as long as 13 years in aqueous Kaiserling solution. Best's carmine stain<sup>5</sup> for glycogen is uniformly nega-

<sup>3</sup> McManus, J. F. A., *Stain Technology*, 1948, **23**, 99.

<sup>4</sup> Hotchkiss, R. D., *Arch. Biochem.*, 1948, **16**, 131.

<sup>†</sup> Material is unavailable from one case.

Table I, the individual titers are remarkably uniform and have values 1 to 2.5 times as great as those of the commercial pool. Titers similar to these were obtained on all other occasions when individual milks or pools were tested. Accordingly, one may conclude that inhibitory activity is a characteristic property of cow's milk.

*Heat stability of the inhibitor.* Samples of undiluted commercial RSM, prepared by centrifugation, were heated in a boiling-water bath for varying periods, cooled, and titrated for residual inhibitory activity against heated swine influenza virus. Raw and pasteurized samples of whole and skim milk were also tested. The results, presented in Table II, show that the inhibitory activity of RSM is similarly reduced by commercial pasteurization (30 minutes at 71°C) and by heating at 100°C for 2 minutes. Heating at 100°C for periods greater than 2 minutes produces very little further change. Pasteurization of raw whole milk, carried out by a different process (20 seconds at 72°C), has little effect on the inhibitor.

From the results with heated RSM, it would appear that the inhibitory activity is associated either with a single substance which has both heat-labile and heat-stable aspects, or with two substances which differ in heat stability. It is interesting that the gradient of inhibition is much more shallow with heated than with unheated skim milk (Table II). On the assumption that the inhibitory activity of milk is associated with a single substance, this difference in gradient is interpreted to mean that the inhibitor becomes weaker, rather than simply more dilute, during heating.<sup>9</sup> Unpublished experiments with the egg-white inhibitor, which is remarkably thermostable, have shown a similar weakening of the inhibitor only after prolonged heating (1 hour or more) at 100°C.

*Mechanism of inhibition.* To determine whether the inhibitor in milk acted on the virus or on the red cells, an experiment was carried out in which RSM dilutions were incubated for varying periods with heated swine virus or with red cells before the second re-

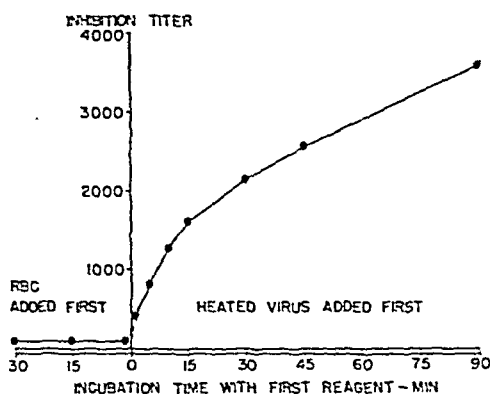


FIG. 1.

Inhibition titer as a function of the order of addition of chicken red blood cells and heated swine influenza virus to dilutions of raw skim milk and the time of incubation at 27°C with the reagent added first.

agent (red cells or virus) was added. Except for this modification, the general set-up of the tests was like that of the routine inhibition titrations. In all cases readings were made one hour after the second reagent was added. The results, plotted in Fig. 1, show that, when RSM dilutions are first incubated with red cells, the inhibition titer is low and independent of time of incubation. In contrast, when RSM dilutions are first incubated with heated virus, the titer, initially low, rises to a high level as incubation is prolonged. This sort of result has been obtained also in similar experiments with egg-white<sup>3</sup> and saliva.<sup>10</sup> It may be concluded that the inhibitor in milk is effective through its capacity to combine with virus.

*Comparison of heated and unheated virus.* Francis<sup>11</sup> showed that the inhibitory effect of normal serum is greater against suitably heated than against unheated virus. This difference in susceptibility of heated and unheated virus to inhibition, explained in terms of a capacity of unheated virus to destroy inhibitor and the loss of such capacity on heating<sup>1,2</sup> has been observed also with milk. Thus, when unheated swine virus was incubated for varying periods with RSM dilutions before the addition of red cells, the conventional inhibition titer rose to about 64 after 5 minutes

<sup>9</sup> Lanni, F., and Beard, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1949, 71, 116.

<sup>10</sup> Seltsam, J. H., Lanni, F., and Beard, J. W., *J. Immunol.*, in press.

<sup>11</sup> Francis, T., Jr., *J. Exp. Med.*, 1947, 65, 1.

TABLE I.  
Inhibitory Activity of Raw and Pasteurized Skim Milk Against Hemagglutination by Heated Swine Influenza Virus.

Cow	Type of milk	Titer
1	Raw	4100
2	"	5100
3	"	4100
4	"	6400
5	"	2600
6	"	6400
7	"	6400
8	"	5400
Commercial pool	"	2600
"	Pasteurized*	460†

\* 30 min. at about 71°C by a vat process.

† Conventional endpoint. The slope of the inhibition curve was very shallow. See Table II and its discussion.

Routine inhibition titrations were performed by the constant virus-varying inhibitor technic,<sup>8</sup> employing 3 to 4 hemagglutinating doses (HD) of purified swine influenza virus,<sup>3</sup> which had been heated for 30 minutes at 53°C. In these routine tests virus was first incubated with inhibitor for 30 minutes, then red cells were added, and readings were made after an additional hour at room temperature. The inhibition titer of a fluid is expressed as the reciprocal of the final dilution of the fluid in the reaction mixture (composed of 0.5 ml inhibitor dilution, 0.5 ml virus suspension, and 1.0 ml 2% chicken red blood cells) giving the standard two-plus (++) endpoint of hemagglutination. Buffered saline, consisting of 0.81% sodium chloride and 0.005 M phosphate at pH 7.3, was the diluent throughout. Details of other types of inhibition tests are given with the description of the experiments.

*Experimental. Survey.* Samples of RSM, prepared from the individual milks of eight cows, were tested for inhibitory activity against heated swine influenza virus, with the results shown in Table I. Samples of a commercial pool of skim milk before and after pasteurization were included.† As seen in

<sup>8</sup> Lanni, F., Sharp, D. G., Eckert, E. A., Dillon, E. S., Beard, D., and Beard, J. W., *J. Biol. Chem.*, 1949, **179**, 1275.

† The commercial samples studied in the present investigation were obtained from Durham Dairy Products, Inc., through the courtesy of Mr. V. J. Ashbaugh, Mgr.

TABLE II. Effect of Commercial Pasteurization and of Heating at 100°C on the Inhibitory Activity of Milk Against Hemagglutination by Heated Swine Influenza Virus.

Milk sample	Reciprocal of final dilution of sample					
	40	80	160	320	640	1,280
Pool 1, raw whole milk	0	0	0	0	0	1
1, after skimming	0	0	0	0	0	1
1, skimmed and pasteurized*	1	2	2-2/3	2	2/3	3
2, raw whole milk	0	0	0	0	0	1
2, pasteurized†	0	0	0	0	1	1
RSM,† 2 min. at 100°C	1	2	2	2/3	3	3
" 5 " "	1	2	2	2/3	3	3-3/4
" 10 " "	1	2-2/3	2	2/3	3	3/4
" 15 " "	1	2-2/3	2	2/3	3	3/4
" 30 " "	1	2-2/3	2	2/3	3	3/4
" 60 " "	2-2/3	2	2	2/3	3	3/4
" 30 min. at about 71°C (160°F) by a vat process.	2-2/3	2	2	2/3	3	3/4
† 20 sec. at about 72°C (162°F) by a continuous flow process.	2-2/3	2	2	2/3	3	3/4

The symbols ± to ++, usually employed to denote degree of hemagglutination, are replaced for convenience by the symbols 1— to 4. The concentrations of red blood cells in the standard suspensions which are assigned these designations have been shown in a previous publication (Fig. 1 of 10).

† Raw skimmed milk obtained from Pool 1.

TABLE IV.  
Properties of Fractions Obtained from Raw Skim Milk (RSM).

Fraction (See text)	Inhibition titer*	Nitrogen,* γ per ml	Purification factor†	Activity partition, %
RSM	5,000	5,280	1.00	100
Casein	410	3,830	0.11	8.2
Whey	4,300	1,130	4.0	86
P50	4,200	213	21	84
P60	300	55	5.8	6.0
P100	24	359	0.07	0.5
S100	11	159	0.07	0.2

\* Calculated to the original volume of RSM.

† Calculated from the titer-nitrogen ratio with reference to the titer-nitrogen ratio of RSM.

when neutral whey is half-saturated with  $(\text{NH}_4)_2\text{SO}_4$ . Less than 1% of the inhibitor is precipitated from neutral whey at 0.33 saturation and less than 10% at 0.4 saturation.<sup>‡</sup>

An example of the results of fractionation is given in Table IV. A volume of 10 ml N acetic acid was added slowly with vigorous stirring to 100 ml pooled RSM, the pH falling from 6.6 to 4.6. The precipitated casein was obtained by centrifugation, washed with 100 ml 1% NaCl, triturated with 2 ml 10% NaOH, taken up in 100 ml 1% NaCl, and neutralized with N acetic acid. The supernatant whey was neutralized with 2.5 N NaOH and refrigerated for 2 days, during which a slight precipitate formed. This precipitate, which was found to contain about 1% of the initial activity and of the initial nitrogen, was centrifuged off and discarded. The supernatant was brought successively to 0.5, 0.6, and 1.0 saturation with solid  $(\text{NH}_4)_2\text{SO}_4$ , added slowly with vigorous stirring. The precipitates, designated P50, P60, and P100, respectively, were taken up in  $\text{H}_2\text{O}$  and dialyzed exhaustively through Visking casing against repeated changes of buffered saline in the cold. The final supernatant at 1.0 saturation, designated S100, was dialyzed against running tap water overnight and then exhaustively against buffered saline in the cold. All of the fractions were analyzed for inhibitor by the routine method and for nitrogen by direct nesslerization.<sup>14</sup> The

results show that the inhibitor can be purified about 20 times, with excellent recovery, by this method. These experiments are being continued.

*Discussion.* The non-dialyzability of the inhibitor and its precipitability by salt suggest that the inhibitor is itself a high molecular weight substance, presumably a protein, or is associated with such a substance.

In its reactions with virus, the milk inhibitor displays the characteristic features of other recently discovered inhibitors, such as those occurring in normal serum, normal allantoic fluid, and egg-white. Thus the inhibitor is more effective against heated than against unheated virus and appears to be susceptible to inactivation by the latter. For this reason, and since all samples of raw milk which have been tested possess roughly the same inhibitory activity, it may be concluded that the inhibitor is a normal component of milk rather than a specific antibody produced in response to casual antigenic stimulation. Experiments now in progress in this laboratory indicate that both the milk and the egg-white inhibitors can be distinguished readily in inhibition experiments from the specific antibody which occurs in convalescent anti-influenza swine serum. For example, antibody is equally effective against heated and unheated viruses, while the other inhibitors are considerably more effective against heated virus. Moreover, the gradient of inhibition in the end-point region is much steeper with antibody than with milk or egg-white.

In view of the reported relation between other naturally occurring inhibitors and mucoproteins,<sup>1,2,4,6,8</sup> it is interesting to note that a mucoprotein, designated *lactomucin*, has

<sup>‡</sup> A solution containing 7.557 equivalents of  $(\text{NH}_4)_2\text{SO}_4$  per liter is regarded as a saturated solution.<sup>15</sup>

<sup>14</sup> Lanni, F., Dillon, M. L., and Beard, J. W., submitted for publication.

TABLE III. Evidence of Inhibitor Destruction by Unheated Influenza Virus A (PR8 Strain) and Lack of Destruction by Heated Virus.

Virus PR8	Incubation with whey*, min.	Total virus nitrogen present, $\gamma$									
		4	2	1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Unheated	Control†	4	4	4	4	4	3/4	3—/3	2—	0/1—	0
	1	4	4	3	3—	2—	0/1—	0	0	0	0
	7.5	4	4	3	2/3—	1	0/1—	0	0	0	0
	15	4	4	3/4—	2/3—	1	0/1—	0	0	0	0
	30	4	4	4—	3—	1	0/1—	0	0	0	0
	60	4	4	4	3	2—	0/1—	0	0	0	0
Heated†	Control†	4	4	4	4	3	1—	0	0	0	0
	1	4	3	4	4	4	3/4—	2/3—	2	0/1—	0
	7.5	4—	3—	2/3—/2	2—	1	0	0	0	0	0
	15	3/4—	2/3—	2—	0/1—	0	0	0	0	0	0
	30	3/4—	2	1—	0/1—	0	0	0	0	0	0
	60	3	2	1—	0	0	0	0	0	0	0
	120	3	2—/2	0	0	0	0	0	0	0	0

\* Final dilution of whey 1:20 in all tubes except controls.  
† Saline substituted for whey.

† Heated for 30 min. at 53°C at a concentration of 8  $\gamma$  N per ml.

of incubation, and then fell progressively, reaching the value 35 after 90 minutes of incubation. After 30 minutes of incubation, the titer was 48; the corresponding titer against heated virus (Fig. 1) was 2150, about 45 times as great. The decrease in titer with unheated virus after 5 minutes of incubation is interpreted as evidence of inhibitor destruction by virus.

*Inhibitor destruction by virus.* Further evidence of inhibitor destruction by virus is presented in Table III, which shows the results of an experiment carried out with whey and influenza virus A (PR8 strain), purified as previously described.<sup>12</sup> The whey was prepared by bringing RSM to pH 4.6 with N acetic acid, filtering off the precipitated casein, and neutralizing the filtrate to pH 7.0 with NaOH. Progressive dilutions of unheated virus were incubated in constant volume with a constant amount of whey for varying periods at room temperature before red cells were added. The results (Table III) show a slight initial increase in inhibition followed by a progressive decrease, manifested in the partial emergence of the hemagglutinating activity of the virus. Almost identical results were obtained with a partially purified fraction, prepared by half-saturating the whey with  $(\text{NH}_4)_2\text{SO}_4$  (see below); and similar results were obtained with RSM and unheated swine influenza virus. The interesting inhibition optimum seen in Table III has been observed also with saliva<sup>10</sup> and has been explained as a consequence of two oppositely manifested reactions.

Table III shows also the results of a similar experiment carried out with whey and heated PR8 virus. With this virus the inhibition increases progressively as incubation with whey is prolonged, the results emphasizing the difference in behavior of heated and unheated virus noted above.

*Partial purification of the inhibitor.* Preliminary experiments directed at fractionation have shown that the milk inhibitor occurs in the whey and can be recovered almost quantitatively in the precipitate which forms

<sup>12</sup> Taylor, A. R., Sharp, D. G., Beard, D., Beard, J. W., Dingle, J. H., and Feller, A. E., *J. Immunol.*, 1943, **47**, 261.

TABLE I.  
Concentration of the Labile Factor in Normal Rabbit Plasma.\*

Stored human plasma, cc Rabbit deprothrombinized plasma, cc		.099 .001	.095 .002
		Prothrombin time (in sec.)	
Rabbit	1	14	12
"	2	14	12
"	3	13½	12
"	4	14	11
"	5	14½	12
"	6	13½	12
"	7	14½	12½
"	8	13	11
"	9	14	12
"	10	14	12

\* The stored human plasma had a prothrombin time of 45 sec. The rabbit plasma was treated with tricalcium phosphate gel (0.012 M) to remove prothrombin.

TABLE II.  
Effect of Dicumarol Treatment on Concentration of the Labile Factor in Rabbits and Dogs.

Animal	Day of treatment	Prothrombin time of whole plasma (sec)	Prothrombin time (sec) of mixture: Stored human plasma, i cc Deprothrombinized plasma of animal treated, ‡ cc	
			.099 .001	.095 .002
Rabbit 1*	0	7	13	12
	1	11	13	11½
	2	8½	13½	11
	3	8½	13	11
	4	10	12½	10½
	5	12	12½	11
Rabbit 2*	0	6½	13½	12
	1	13	14	11½
	2	20	13	11
	3	27	13	11
	4	53	13	11
	5	53	13	11
Dog 1	0	6	20	16
	1	11	22	15
	2	17	19	14
	3	25	18	15
	4	30	19	15½
Dog 2	0	6	18	14
	1	12	22	16½
	2	17	20	14½
	3	27	22	16
	4	40	20½	16

\* The data were selected to show that the sensitivity to dicumarol bears no relation to variations of the labile factor.

† The stored human plasma had a prothrombin time of 45 sec.

‡ Rabbit and dog plasma were treated with tricalcium phosphate gel (0.012 M) prior to use, to remove prothrombin.

be accounted for by the methods for determining the labile factor (Ac-globulin). With the method of Ware and Seegers<sup>8</sup> the variation of the labile factor in rabbit plasma was found to be from 92 to 310 units and in dogs from 158 to 203 units.<sup>9</sup> In contrast, the method of Quick and Stefanini showed that the labile factor in rabbit plasma was relatively con-

<sup>8</sup> Ware, A. G., and Seegers, W. H., *J. Biol. Chem.*, 1948, **172**, 699.

<sup>9</sup> Murphy, R. C., and Seegers, W. H., *Am. J. Physiol.*, 1948, **154**, 134.

been isolated from milk<sup>13</sup> and that the reported solubility properties of this substance are roughly those of the inhibitor. The relation between the milk inhibitor and lactomucin is being investigated.

**Summary.** Milk is capable of inhibiting hemagglutination by influenza viruses, the

inhibitory effect being greater against heated than against unheated viruses. The inhibitor, which is non-dialyzable and moderately heat stable, occurs in the whey and can be salted out by half-saturation with ammonium sulfate. Evidence is presented that the inhibitor is a characteristic component of milk rather than a specific antibody.

<sup>13</sup> Sorensen, M., and Sorensen, S. P. L., *Compt. Rend. Trav. Lab. Carlsberg, Sér. Chim.*, 1938-41, **23**, 55.

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## Effect of Dicumarol on Concentration of the Labile Factor.\* (17390)

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The observation that the loss of prothrombin activity in stored human plasma could be restored by adding to it rabbit or dog plasma which was markedly depleted of prothrombin by means of dicumarol, led to the discovery of the labile factor and to the assumption that this agent is not affected by dicumarol.<sup>1</sup> The development of a quantitative procedure for estimating the labile factor in blood,<sup>2</sup> now makes it possible to test the validity of this assumption.

**Experimental.** Rabbits and dogs were given dicumarol orally (5 mg per kilo body weight daily) until the prothrombin time became markedly prolonged. The prothrombin activity was determined by the original one-stage procedure.<sup>3</sup> For the estimation of the concentration of the labile factor, the method of Quick and Stefanini<sup>2</sup> was employed. The method consists essentially in determining the prothrombin time of stored plasma after the addition of a measured amount of the plasma to be tested which has been deprothrombinized by adsorption with tricalcium phosphate. The reduction of the prothrombin time is a measure of the concentration of the labile factor.

**Results.** From the data in Table I it can be seen that the concentration of the labile factor in normal rabbit plasma is remarkably constant. This is also true of dog plasma, but the concentration is only one-fifth that of rabbit plasma.<sup>2</sup> When dicumarol is administered the prothrombin activity as measured by prothrombin time decreases progressively and after one week may reach a level below one per cent of normal (Table II). In spite of this drastic decrease, the concentration of the labile factor shows no greater fluctuation than may be attributed to the inherent variations of the method. It can be concluded that dicumarol does not influence the labile factor in rabbits and dogs. Preliminary studies indicate that this is also true in humans.

Owren,<sup>4</sup> Fantl and Nance<sup>5</sup> and Seegers and Ware<sup>6</sup> agree at least tentatively that their respective agents, factor V, accelerator factor and Ac-globulin are the same as the labile factor. The finding of Fahey, Olwin and Ware<sup>7</sup> that dicumarol causes at least a moderate decrease in Ac-globulin in dogs is not in agreement with the present results. It is likely that the difference in findings can

\* This investigation was supported by a grant from the Division of Research Grants, National Institute of Health.

<sup>1</sup> Quick, A. J., *Am. J. Physiol.*, 1943, **140**, 212.

<sup>2</sup> Quick, A. J., and Stefanini, M., *J. Lab. Clin. Med.*, 1948, **33**, 819.

<sup>3</sup> Quick, A. J., *J.A.M.A.*, 1938, **110**, 1658.

<sup>4</sup> Owren, P. A., *Biochem. J.*, 1948, **43**, 136.

<sup>5</sup> Fantl, P., and Nance, M. H., *Med. J. Australia*, 1948, **1**, 98.

<sup>6</sup> Seegers, W. H., and Ware, A. G., *Am. J. Clin. Pathol.*, 1949, **10**, 441.

<sup>7</sup> Fahey, J. L., Olwin, J. N., and Ware, A. G., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 491.



FIG. 1.

Gomori's stain for alkaline phosphatase. Prominent staining of bile capillaries in the normal rabbit liver. Accumulation of granular phosphatase around bile canaliculi. Note intracellular branches.  $\times 1400$ .



FIG. 2.

Dilatation of bile capillaries 4 days after biliary obstruction. Note intracellular network.  $\times 1400$ .

and Furth.<sup>5</sup> Sections were incubated for 2 hours.

**Results.** The distribution of alkaline phosphatase activity in the normal liver of the rabbit was quite similar to that previously described in the dog liver.<sup>5</sup> Surrounding the distinctly outlined bile capillaries, granular deposits of alkaline phosphatase were often seen.<sup>6</sup> Fig. 1 shows an intercellularly located bile capillary situated between 2 liver cell cords. From this axial canaliculus, not only intercellular blind ending branches, but also

short intracellular ramifications are seen to spring off. These intracellular branches often stain as black lines but occasionally show a distinct lumen. By using the fine adjustment screw under oil immersion, one can be reasonably sure that the short branches lie really within the cell and do not represent portions of communicating intercellular ramifications. Although very short, knob like outpouchings are quite common, larger intracellular branches, as pictured in Fig. 1 are not too frequent in the normal liver. In livers of rabbits which survived the experimental biliary obstruction from 4 to 31 days, however, there occurred not only marked dilation and tortuosity of bile capillaries but intracellular

<sup>5</sup> Kabat, E. A., and Furth, J., *Am. J. Path.*, 1941, 17, 303.

<sup>6</sup> Deane, H. W., and Dempsey, E. W., *Anat. Rec.*, 1945, 93, 401.



stant, and was 5 times higher than in dog plasma.

These differences in results are probably traceable, at least in part, to technical factors of the methods. It appears that the procedure employed in this study is more sensitive and perhaps subject to less error. Nevertheless, when the two methods are applied to normal plasmas they yield results which roughly parallel each other, and both show a low concentration of labile factor in human blood. More important but far more difficult to harmonize and discuss critically are the differences in views concerning the action of the labile factor. Owren, Fantl and Seegers look upon this factor as an accelerator or catalyst, while the writers conclude that it reacts stoichiometrically since a manifold increase in its concentration does not alter the prothrombin time nor the speed of prothrombin conversion.<sup>10</sup> Recently findings have been made which clearly suggest that prothrombin in human blood exists partly in an active

form and partly in a precursor state.<sup>11</sup> This discovery introduces a new element in the clotting mechanism requiring evaluation. It is likely that the concept that prothrombin *per se* can change its convertibility or that catalysts can alter it, will require reinterpretation. Since the one-stage method measures active prothrombin, and the two-stage probably total prothrombin, findings such as those reported by Owen and Bollman<sup>12</sup> that the two methods when applied to dicumarolized blood showed marked difference in prothrombin level can be explained without postulating a factor which changes the rate of conversion of prothrombin to thrombin.

**Conclusions.** The labile factor of the prothrombin complex is relatively constant in normal rabbit and dog blood. It is not reduced by dicumarol even after the prothrombin activity falls to a very low level.

<sup>11</sup> Quick, A. J., and Stefanini, M., *J. Lab. Clin. Med.*, 1949, **34**, 1203.

<sup>12</sup> Owen, C. A., and Bollman, J. L., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 231.

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<sup>10</sup> Quick, A. J., and Stefanini, M., *J. Lab. Clin. Med.*, 1949, **34**, 973.

## Intracellular Bile Canaliculi in the Rabbit Liver. (17391)

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There is no controversy as to the general pattern of the bile canaliculi in the mammalian liver with but one exception. This concerns the existence of intracellular branches.

Gomori's<sup>1</sup> technic for the histochemical demonstration of alkaline phosphatase furnishes a very convenient method for the study of the bile capillaries under normal and abnormal conditions, since the bile canaliculi in the liver of some species show striking staining properties.<sup>2-4</sup> The results obtained with

this method in the liver of rabbits under normal conditions and after experimental biliary obstruction, strongly suggest the existence of intracellular branches.

**Material and Method.** In medium sized rabbit under ether anesthesia, a liver biopsy was taken and the cystic and bile ducts were ligated. Twenty-two animals were allowed to survive from 1 to 31 days. In addition, livers from normal animals were available. Sections from tissues fixed in cold acetone were stained with Gomori's method<sup>1</sup> as modified by Kabat

<sup>1</sup> Gomori, G., *Proc. Soc. Exp. Biol. and Med.*, 1939, **42**, 23.

<sup>2</sup> Gomori, G., *J. Cell. and Comp. Physiol.*, 1941, **17**, 71.

<sup>3</sup> Wachstein, M., and Zak, F. G., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 73.

<sup>4</sup> Jacoby, F., *J. Phys.*, 1947, **100**, 23P.

Leading investigators in this field, including Long,<sup>2</sup> Lurie, and Rich, have recognized that the mechanism of caseation and softening is still the key problem in tuberculosis for, as is well known, in the centers of caseous areas tubercle bacilli tend to die, whereas in areas of softening they multiply profusely. As Jaffe<sup>4</sup> expressed it, "The rapid liquefaction of the caseated infiltration is very dangerous, since it opens blood vessels and bronchi. Hemorrhage and aspiration result and the infection spreads rapidly to other portions of the lung." The question arises how does necrotic, caseous tissue in tuberculosis differ from similar material seen in abscess, gumma or infarct? The answer is not yet at hand. Opie and Barker<sup>5</sup> investigated this problem, employing hypertrophied tuberculous lymphatic glands, removed from experimentally infected dogs, as source of enzyme and 50% blood serum, previously heated to 75°C as substrate. They concluded, "When during the third week after inoculation of tubercle bacilli, the figures indicate a maximum degree of digestion, caseation is beginning. . . . After onset of caseation there is gradual and probable complete disappearance of enzymes." Rich<sup>1</sup> summarizes the situation as follows, "At present it remains a problem whether the failure of autolysis to occur in caseous areas is due to an inadequate content of proteolytic enzymes in the monocytes or to the presence of substances that inhibit or destroy the enzymes at the site."

We have studied the behavior of Cathepsin II during various stages of caseation since this endocellular proteolytic enzyme (BA-amidase)<sup>6</sup> is probably concerned in tissue breakdown and in protein synthesis. If this or similar enzymes should play a significant role in the mechanism of caseation and/or softening, there is hope that their action may be

influenced by known activators or inhibitors.

**Experimental procedures.** In order to produce caseous tissue, albino rabbits were infected with tubercle bacilli as follows: Animals of Group 1 were injected intratracheally, without previous immunization, with a dose of 0.001 mg of a virulent Ravenel culture of tubercle bacilli. Those of Group 2 were first immunized by means of 2 successive weekly intravenous injections of 1 mg of a lowly virulent R1 strain of *M. tuberculosis* and then 2 weeks later injected intratracheally in the same manner as the animals in Group 1. They were x-rayed at frequent intervals in order to follow the progress of the infection. Five to 8 animals of each group were killed at varying intervals of time—from 5 to 24 weeks after intratracheal infection, by exsanguination from the carotid artery. The organs (lungs, kidneys, liver, and spleen) were removed, weighed, and small sections taken for histologic study. The remainder of the tissues were frozen intact in a dry ice chamber and aliquots (15-20 g) were chipped in the frozen state and placed, together with 10 times their weight of ice water containing chipped ice, into a Waring blender and homogenized. Under these conditions the blender remained ice-cold during the one or 2 minutes of homogenation. Aliquots of the homogenates were used for semi-micro-Kjeldahl determinations and enzyme studies. Enzymatic hydrolysis of BAA (benzoylarginineamide) was estimated by the method of Greenstein and Leuthardt,<sup>7</sup> which determines the amount of ammonia N liberated. The reaction constant was calculated from the

equation  $K = \frac{1}{t} \log \frac{100}{100-x}$  where x is percent-

age hydrolysis and t is time in hours. The activity was expressed in units per mg of nitrogen. Under the conditions of these experiments the first order reaction constant for the liberation of ammonia from BAA was proportional to the amount of homogenate used and hence to the enzyme (BA-amidase) concentration.<sup>8</sup>

In carrying out the tests 1.0 ml of organ homogenate was added to 1.0 ml solution of

<sup>7</sup> Greenstein, J. E., and Leuthardt, F. M., *J. Nat. Cancer Inst.*, 1945-46, 6, 203.

<sup>2</sup> Long, E. R., *Arch. Pathol.*, 1929, 28, 719.

<sup>4</sup> Jaffe, R. H., *Arch. Pathol.*, 1934, 18, 712.

<sup>5</sup> Opie, E. L., and Barker, R. L., *J. Exp. Med.*, 1902, 10, 645.

<sup>6</sup> Bergmann, M., A Classification of Proteolytic Enzymes, in "Advances in Enzymology," edited by P. P. Nord and C. H. Werkman, Interscience Publ., Inc., New York, 1941, 1, 63-92, and 1942, 2, 49-68.

branches also became quite numerous. Fig. 2 depicts a network of bile canaliculi located within 2 liver cells. The intracellular branches are seen to possess a real lumen.

**Discussion.** In most modern textbooks of histology, no mention is made of intracellular bile capillaries. Their possible occurrence is conceded in Sharpey-Schafer's<sup>7</sup> and Stöhr's textbooks.<sup>8</sup> Their existence, however, is denied by Maximow and Bloom.<sup>9</sup> The study of finer morphological details with the phosphatase staining technic is considerably helped by the fact that in contrast to the dog liver, the rabbit liver, after biliary obstruction shows no appreciable increase of cytoplasmic phosphatase activity such as often blurs the outline of the dilated capillaries. Clara,<sup>10</sup> using

special staining technics, considers the occurrence of intracellular branches in the rabbit liver as likely, but not as definitely, proved. He pointed out that an intracellular location may seemingly be present, while in reality it is due to a location of bile capillaries on the surface of the liver cells in question. While the possibility of this cannot be completely excluded, in the case of the normal rabbit liver, it is quite unlikely in that of the liver after biliary obstruction. Here, a network of dilated bile capillaries is frequently observed on the same plane within a single liver cell. Occasionally, it is found at the level of the cell nucleus.

**Summary.** With the aid of the histochemical phosphatase stain, intracellular bile canaliculi can be demonstrated in the normal rabbit liver. These intracellular branches become considerably more pronounced following experimental biliary obstruction.

<sup>7</sup> Sharpey-Schafer, E., *Essentials of Histology, Descriptive and Practical for use of students*. 13th edition by M. Carleton, London, New York, Toronto.

<sup>8</sup> Stöhr, P., *Lehrbuch der Histologie und der Mikroskopischen Anatomie des Menschen*, 25th edition by W. V. Mollendorf, Jena 1943, p. 349.

<sup>9</sup> Maximow, A. A., and Bloom, W., *Textbook of Histology*, Philadelphia and London, 1947, p. 43.

<sup>10</sup> Clara, M., *Z. F. Mikr. Anat. Forsch.*, 1934, **352**, 1.

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## Enzymatic Hydrolysis of Benzoylarginineamide by Normal and Tuberculous Tissue of Rabbits.\* (17392)

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Caseation has been described by Rich<sup>1</sup> in these words, "When, as a result of partial autolysis, necrotic cells lose their structure and outlines, and their remains together with

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<sup>1</sup> Rich, A. R., *The Pathogenesis of Tuberculosis*. C. C. Thomas, Publ., Springfield, Ill., 1946, p. 733 ff.

intercellular materials tend to become fused into a formless, coagulated and more or less inspissated mass, the process is termed caseation." In rabbits, experimentally infected with virulent tubercle bacilli, caseation is usually observed about the third week of the disease, when according to Lurie,<sup>2</sup> the monocytes, which make up the bulk of the cellular material in the caseous area, have been transformed into epithelioid cells and allergy to tuberculin has developed. This suggests that the process of caseation is associated with some degree of acquired tissue immunity or resistance to infection with tubercle bacilli.

<sup>2</sup> Lurie, M. B., *J. Exp. Med.*, 1933, **57**, 181.

TABLE II.  
 Illustrative Analytical Data on Normal and Infected Rabbits.

Rabbit No.	Body wt	Organ	Organ wt	% N	K $\times 10^3$ /ml. hom.	K/g N
Normal animals						
9	3896	Li†	78.3	2.87	52.2	19.3
		K	13.0	2.53	14.7	14.8
		S	1.39	2.37	16.0	25.8
		Lu	11.0	2.02	12.8	8.1
13	4140	Li	96.0	2.72	57.7	20.0
		K	18.9	2.39	22.2	15.7
		S	1.59	2.11	12.0	17.6
		Lu	11.74	2.12	16.1	8.4
18	2645	Li	98.0	2.23	58.6	18.0
		K	12.8	2.53	22.4	15.8
		S	1.04	3.41	3.1	8.1
		Lu	8.7	2.51	14.4	6.2
26	2870	Li	97.3	2.55	60.5	16.9
		K	12.8	2.46	31.2	22.0
		S	1.02	1.76	5.6	13.6
		Lu	9.36	2.22	11.2	6.5
Infected animals*						
1	3286	Li	95.6	2.81	64	24.4
		K	24.3	2.34	85	28.5
		S	2.7	1.48	31	39.0
		Lu	131.0	1.80	16.3	12.5
2	2990	Li	64.0	2.61	30.8	18.5
		K	16.9	2.60	49.8	22.8
		S	1.4	2.60	18.2	12.4
		Lu	110.0	2.26	16.9	10.1
5	2590	Li	86.8	2.35	—	—
		K	25.6	2.17	45.9	21.1
		S	2.32	2.09	57.0	26.0
		Lu	18.2	2.02	11.0	4.5
6	2665	Li	73.0	2.76	45.1	21.6
		K	15.3	2.25	45.9	27.0
		S	2.0	2.14	20.3	15.7
		Lu	75.3	2.12	3.6	2.6
8	3296	Li	80.9	3.06	46.6	19.7
		K	24.6	2.17	91.1	25.5
		S	1.56	2.58	50.8	19.0
		Lu	26.3	1.71	43.0	25.8

\* Rabbits killed 16 wk after primary infection with virulent tubercle bacilli.

† Li = Liver; K = Kidney; S = Spleen; Lu = Lung; hom. = Homogenate.

packed with lymphocytes and young fibroblasts. The surrounding lung tissue was fairly normal. There were a few RBC but no fluid. No PMN were seen and not all of the endothelial cells were desquamated. The liver showed no tubercles. There was some congestion and cloudy swelling, also periductal proliferation with monocytes and young fibroblasts. The hepatic lobules were fairly well demarcated as a result of the interstitial reaction. In sections of the kidneys there were

many areas of caseation necrosis surrounded by large giant cells. There were also closely-packed collections of cells (monocytes, lymphocytes, and young fibroblasts), the centers of which showed softening. Congestion, cloudy swelling and beginning desquamation of the tubular epithelium were evident in addition to many giant cells. The spleen showed large areas of necrosis with disintegration of the nuclei and absence of cellular outlines. The Malpighian follicles were prominent.

TABLE I.  
Comparison of Benzoylarginine Amidase Activity of Organs of Rabbits Killed at Various Time Intervals After Primary or Reinfection with Virulent Tubercle Bacilli.

Group	When killed	No. of rabbits	Liver K/g N			Spleen K/g N			Kidneys K/g N			Lungs K/g N		
			Avg.	S.E.	Sign	Avg.	S.E.	Sign	Avg.	S.E.	Sign	Avg.	S.E.	Sign
A	Normal controls	14	28	2.1		24	2.7		24	1.9		8.6	0.8	
After primary infection														
B	5 wk	5	20	1.1	+	21	3.1	—	20	1.7	++	9.8	1.4	—
C	16 wk	5	21	0.9	±	31	6.9	—	25	1.4	—	11.7	4.1	—
After reinfection														
D	5 wk	5	26	1.9	—	31	2.4	—	30	4.1	—	17.2	2.2	++
E	16-20 wk	5	17	2.1	++	27	4.2	—	38	6.0	++	21.0	2.9	++

S.E. = Standard error ( $\pm$ ). Sign—indicates the significance of the experimental averages when compared with the controls. A minus (—) means that the Fischer "t" test gave a "P" value of 0.05 indicating probable significance. A plus (+) denotes a "P" value greater than 0.02 and less than 0.05, indicating statistical significance. Two plus (++) indicates values less than 0.02 or a high degree of significance.

0.1 molar citrate buffer (pH 5.1) containing 0.02 mmols of BAA and 0.01 mmols of cysteine hydrochloride. Digestion was carried out in large test tubes in a 39° bath. After 2 and 4 hours, the tubes were removed, 1 ml of saturated potassium carbonate and 3 ml of water were added and the whole aerated as described by Greenstein and Leuthardt. The ammonia liberated was absorbed in a 2% boric acid solution containing indicator (methyl red-bromocresol green) and titrated with N/14 HCl. A correction blank for zero time was set up in tubes which were treated immediately with saturated  $K_2CO_3$ .

**Pathologic findings.** The rabbits were killed by exsanguination, at various time intervals after infection, as indicated in Tables I-III. At autopsy the kidneys and lungs showed varying degrees of caseation. Microscopically, these areas were surrounded by inflammatory cells or granulation tissue. There was no caseation in the livers or spleens. In animals which were killed 5 and 16 weeks after primary infection, the lungs were markedly enlarged and weighed from 25 to 135 g as contrasted with an average normal of 10 g. Two protocols are given to illustrate the pathologic findings.

**I. Pathologic findings after primary infection.** Rabbit 8, killed 16 weeks after infection with a virulent Ravenel culture, showed partial consolidation of the lower lobe of the left lung. There were numerous scattered caseous tubercles from 2 to 5 mm in diameter on the surfaces and also within the parenchyma of

the lung. The cortices of the kidneys were studded with numerous, firm gray, elevated tubercles, each 2 to 4 mm in diameter. The spleen and liver showed no gross changes.

Microscopically, there was extensive caseation in both lungs and kidneys. The latter also showed extensive necrosis. There was evidence of hemorrhage and some tuberculous involvement in the spleen.

**II. Pathologic findings after reinfection.** Rabbit 66 was reinfected June 1, 1948 after having received 2 intravenous immunizing doses of an R1 culture of *M. tuberculosis* and killed 3 months after intratracheal infection. X-rays, made 6/9/48 and 8/18/48, showed in the fourth interspace of the right lung several areas of density, peripheral to the heart shadow, suggesting consolidation. The remainder of the right lung presented no abnormalities. In the left lung, the bronchial shadows at the hilum of the lung were accentuated. In the fourth interspace there were several spots suggestive of foci of consolidation.

**Gross anatomic findings.** The lungs were well aerated. Several small tubercles were seen on the pleural surfaces. The surface of each kidney showed a single large, prominent tubercle. The spleen was enlarged and showed several tubercles on the surface. The liver was not involved.

**Histological findings.** In sections of the lungs small miliary tubercles were seen whose centers stained well. At the periphery of one tubercle there was an area which was densely

protein nitrogen (Table III). In spite of this increase in organ weight and the possibility of dilution of enzyme, the rates of hydrolysis of BAA were not significantly different from the normal. Analyses made of bits of caseous material removed and freed from adjacent lung tissue showed them to be enzymatically very active.

The present report confirms the work previously published by Weiss and Halliday<sup>9</sup> that livers and spleens of normal rabbits usually show greater activity of Cathepsin II (BA-amidase) than the lungs. The rate of hydrolysis of lung homogenates is about one-third that of liver. This is of interest since the lungs<sup>2</sup> cannot destroy virulent tubercle bacilli as effectively as do other organs. Considering the size of the liver, it is probably one of the greatest sources of cathepsin in the body.

**Summary and conclusions.** 1. Caseous material was produced in the lungs and kidneys of rabbits by intratracheal infection with a virulent, Ravenel culture of tubercle bacilli. The animals were killed at intervals of from 5 to 20 weeks after infection. The lungs and kidneys, together with the livers and spleens, were removed immediately after exsanguination and death of the animals, preserved at a very low temperature in a dry ice refrigerator, and examined for enzymatic activity.

2. Homogenates of tuberculous lung tissue containing very large amounts of caseous material can hydrolyze benzoylarginineamide (BAA) at pH 5.1 at a more rapid rate than those of homologous normal tissues. The lungs and kidneys of animals which were first immunized with a non-virulent culture before reinfection with a virulent strain of *M. tuber-*

*culosis* were found to have greater enzymatic activity than those with a primary infection.

3. These results are analogous to those of Lurie who showed an increased capacity of rabbit tissue to destroy virulent tubercle bacilli under similar circumstances. They are also of interest since we have previously demonstrated<sup>9</sup> a parallelism between bactericidal action and the rate of hydrolysis of BAA in innate organ immunity.

4. It is not clear whether the observed increased rate of enzymatic activity has its origin in the caseous ("necrotic") material itself or in the surrounding inflammatory exudate ("granulomatous tissue"). The following hypothesis is suggested: The lungs and kidneys of rabbits possess little innate resistance to infection with virulent tubercle bacilli. Hence the latter multiply freely and bring about an inflammatory exudate which is the source of the added enzyme. The liver and spleen, on the other hand, are organs of high natural immunity. They do not permit rapid multiplication of tubercle bacilli (Lurie<sup>2</sup>). The intense inflammatory response with enzyme-laden cells is therefore not called forth.

5. The decreased rate in BA-amidase activity observed in liver homogenates after primary and reinfection, may perhaps be due to the loss of labile liver protein which occurs during the malnutrition accompanying the late stages of a virulent tuberculous infection.

It is a pleasure to acknowledge our indebtedness to Dr. Max B. Lurie of the Henry Phipps Institute of the University of Pennsylvania for his many courtesies; to Dr. David Fishback and to Dr. Helen Ingleby of the Jewish Hospital for the pathologic reports and to Dr. S. Adelman for the x-ray examinations and interpretations.

<sup>9</sup> Weiss, C., and Halliday, N., *J. Immunol.*, 1944, 40, 251.

TABLE III.  
Total Nitrogen, Protein N, and N.P.N. of Tuberculous and Normal Lungs.

Rabbit No.	Total N, mg	Total protein, N	N.P.N., mg	N.P.N. as % of total N
Tuberculous rabbits				
1	2360	1950	410	17.3
2	2500	2190	310	12.4
6	1480	1270	44	14.1
8	446	381	65	14.5
Normal rabbits				
9	222	203	20	9.0
11	265	251	14	5.3
13	248	222	26	8.9
18	221	206	15	6.8

16 weeks following primary infection the animals showed diffuse caseous consolidation involving both lungs almost completely. These data show that most of the increase in N is due to protein and not to N.P.N.

*Results and discussion.* The biochemical data which are summarized in Tables I-III indicate that homogenates of lungs and kidneys of tuberculous rabbits which are diffusely infiltrated with caseous material are enzymatically very active. While there are individual variations, there is, nevertheless, a statistically significant difference in the rates at which these homogenates hydrolyze BAA which depends upon whether we are dealing with tissues from animals with primary infection or with reinfection. In the former, the rate of hydrolysis of lung tissue was not significantly altered as compared with the normal. Likewise, the kidneys showed either normal rates or moderately decreased activities. After reinfection, however, both lung and kidney homogenates revealed marked increases in hydrolytic activity. These data are strikingly similar to the immunologic results of Lurie,<sup>2</sup> who showed that the lungs and kidneys of normal rabbits possess little innate power to destroy virulent tubercle bacilli after primary infection. But as the result of immunization and reinfection there is more complete destruction of these microorganisms. This increase in bactericidal action is associated with an acceleration of the localized inflammatory process and a heightened physiologic activity of the tissue phagocytes. The liver and spleen, having a high innate capacity to destroy virulent tubercle bacilli, do not call forth any appreciable inflammatory response. The observation that liver homogenates have decreased enzymatic activity after primary as well as reinfection may perhaps be ex-

plained by the fact that a loss of liver Cathepsin II may accompany the loss of labile liver protein during the state of malnutrition late in the tuberculous infection.<sup>8</sup> It is also of interest to note that the loss of enzyme activity from the liver takes place earlier following primary infection than following reinfection.

While the data clearly indicate that homogenates of certain organs which contain large amounts of tuberculous caseous material are not enzymatically inert but may show rates of hydrolysis of BAA which are far greater than the corresponding normal values, we have no precise information as to the origin of this added cathepsin. There are 3 possibilities: (a) the inflammatory exudate (or "granulation tissue") which surrounds the necrotic or caseous material is rich in BA-amidase, (b) the caseous material itself, being derived from the breakdown of inflammatory cells is very active enzymatically or (c) the normal, adjacent tissue has acquired an increased rate of hydrolysis. That the first of these two possibilities is the most likely to be true is suggested from the following data:

Ten rabbits which were killed 5 or 16 weeks after primary intratracheal infection with virulent tubercle bacilli had lungs weighing 25 to 125 g, as compared with an average normal value of 10 g. Microscopically, there was caseous and cellular infiltration and chemically there was proof of an accumulation of

<sup>8</sup> Schultz, J., *J. Biol. Chem.*, 1949, **178**, 451.

TABLE I. *In vivo* Acetylation of Tryptophan and Glycine.

Exp. No.	Compounds injected	Cts./min. administered, $\times 1000$	Acetyl amino acid excreted, mg	Activity cts./min./mg acetyl amino acid	Activity cts./min./mg methylene tryptophan
II	Acetyl DL-tryptophan, DL-tryptophan*	100	75	30.7	37.6
III	Acetyl DL-tryptophan, DL-tryptophan, L-tryptophan (100 mg)	100	58	150	164
III	Acetyl DL-tryptophan, DL-tryptophan* DL-tryptophan (200 mg)	100	40	15.8	18.1
IV	Acetyl DL-tryptophan, acetate*	780	49	4.4	5.5
V	Acetyl L-tryptophan, acetate*	780	8.7	3.7	4.1
VI	Acetyl-L-tryptophan, alanine*	530	54	5.0	5.3
VII	Acetyl-glycine, glycine*	240	98†	3.2	

\* Indicates the radioactive compound. † The data represent the average results from 2 rats. In the rest one rat was used.

‡ Final amount recovered pure.

beta-carbon and had an activity of 106,000 counts per minute per mg. The alanine was also the racemic compound labeled on the alpha-carbon (600,000 counts per minute per mg). The acetate and glycine were labeled on the carboxyl carbons (210,000 counts per minute per mg and 1,000,000 counts per minute per mg respectively).

In Experiments I to VI, 100 mg of the acetyl-tryptophan (DL- or L- dissolved in 3.8 ml water (pH 7.4) were injected intravenously by the jugular vein immediately followed by intraperitoneal injections of the other compounds mentioned in Table I. In Experiment VII, the rat was injected intravenously with 300 mg of acetyl-glycine in 3 ml water followed by the labeled glycine solution (0.2 ml) injected intraperitoneally. The urine was collected for the next 8 hours in all cases, the bladder being emptied at autopsy if required.

Acetyl-DL-tryptophan and acetyl-glycine were incubated with labeled tryptophan and glycine respectively for 8 hours in phosphate buffer at pH 7.4 at 37° for 8 hours. The acetyl amino acids isolated from the mixture contained no C<sup>14</sup> showing there was no chemical exchange.

**Results.** The results presented in Table I show that natural amino acids are acetylated *in vivo* in rats. In experiments II and III, the inert L- or DL-tryptophan was added to dilute the specific radioactivity with a view to determining whether the excreted radioactive acetyl derivative was predominantly of the L- or D- configuration. This is of interest since there is no biological mechanism for the deacetylation of D-amino acids.<sup>10</sup> The first four experiments, however, do not definitely decide this question. The subsequent experiments in which acetyl-L-tryptophan was used, leave no doubt that the L-isomer can be acetylated *in vivo*. In the last experiment with acetyl-glycine, the problem of optical isomerism does not exist.

The radioactivity in the isolated acetyl-L-tryptophan and acetyl-glycine is rather low. This does not mean that acetylation of amino acids is necessarily a reaction of little im-

<sup>10</sup> Bloch, K., *Physiol. Rev.*, 1947, **27**, 574.



## *In vivo* Acetylation of Natural Amino Acids. (17393)

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Interest in the acetylation of natural amino acids stems from the suggestion that acetyl-amino acids may be intermediates in protein synthesis.<sup>1</sup> Bloch and Borek<sup>2</sup> demonstrated acetylation of leucine and phenylalanine in liver slices. So far, acetylation of natural amino acids has not been shown *in vivo* probably because in most cases such derivatives are further metabolized rapidly and do not accumulate sufficiently to permit isolation. Knoop<sup>3</sup> found that the unnatural alpha amino acid, phenylamino butyric acid, is readily acetylated in the dog.

Luck and coworkers<sup>4</sup> observed that most of the acetyl-DL-tryptophan administered intravenously to human subjects is excreted unchanged although excretion is negligible when the substance is given orally.<sup>5</sup> Preliminary experiments showed that rats also excrete acetyltryptophan in considerable amounts when it is given intravenously. We have accordingly taken advantage of this behavior to trap any labeled acetyltryptophan formed *in vivo*.

**Experimental methods.** Standard methods were used for the preparation of acetyl derivatives of DL- and L-tryptophan,<sup>6</sup> and acetyl-glycine.<sup>7</sup> Acetyltryptophan was determined colorimetrically by the reaction with p-dimethylaminobenzaldehyde.<sup>4</sup>

**Isolation of acetyltryptophan.** Urine was made alkaline to phenolphthalein and extract-

ed continuously with ether for 24 hours. It was next acidified to congo red and reextracted with ether for another 24 hours. The second ether extract was evaporated to dryness, the residue dissolved in hot water, and decolorized with charcoal. Crystals of acetyltryptophan appeared readily on cooling. Usually, two recrystallizations were enough to give a pure product. Further recrystallizations did not lower the specific radioactivity. The products isolated in experiments I to IV melted at 205-207° (reported 206°) and that in experiments V and VI at 188-189° (reported 190°). The melting points showed no depression on mixing with authentic derivatives. Kjeldahl nitrogen, found 11.28 to 11.46%; theory 11.37%. In order to establish that the radioactivity was due entirely to tryptophan, the isolated acetyltryptophan was hydrolyzed<sup>8</sup> and the convenient formaldehyde derivative of tryptophan prepared.<sup>9</sup> In experiment V 20 mg of acetyl-L-tryptophan was added to the urine as carrier. The specific activity per mole was unchanged (Table I). The derivatives melted sharply at 309-310°. Nitrogen, found 12.88 to 12.99%; theory 12.96%.

**Isolation of acetyl-glycine.** The method was similar except that ethyl acetate was used instead of ether. Crystallizations have to be carried out with greater care since acetyl-glycine is more soluble in cold water than acetyl-tryptophan. The acetyl-glycine was recrystallized to constant specific activity. It melted at 206°, showed no depression in m.p. on mixing with pure acetyl-glycine and had a nitrogen content of 11.87% (theory 11.96%).

The samples were deposited on aluminum discs from water or petroleum ether suspension and counted with a thin mica-window Geiger-Muller Counter.

The tryptophan used in the experiments was a racemic mixture labeled with C<sup>14</sup> on the

\* Government of India scholar.

<sup>1</sup> Rittenberg, D., and Shemin, D., *Ann. Rev. Biochem.*, 1946, **15**, 247.

<sup>2</sup> Bloch, K., and Borek, E., *J. Biol. Chem.*, 1946, **164**, 483.

<sup>3</sup> Knoop, F., *Z. physiol. Chem.*, 1910, **67**, 489.

<sup>4</sup> Luck, J. M., Boyer, P. D., and Hall, V. E., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 177.

<sup>5</sup> Albanese, A. A., Frankston, J. E., and Irby, V., *J. Biol. Chem.*, 1945, **160**, 31.

<sup>6</sup> Du Vigneaud, V., and Sealock, R. R., *J. Biol. Chem.*, 1932, **96**, 511.

<sup>7</sup> Herbst, R. M., and Shemin, D., *Organic Synthesis*, 1939, **19**, 4.

<sup>8</sup> Berg, C. P., *J. Biol. Chem.*, 1933, **100**, 79.

<sup>9</sup> Jacobs, W. A., and Craig, L. C., *J. Biol. Chem.*, 1936, **113**, 759.

TABLE I. Concentration of Bacitracin in Body Tissues and Fluids of 11 Rabbits After a Single Large Intravenous Injection of Bacitracin. (3,000 units/kilo body wt.)

Tissue or fluid Rabbit No.	Units of bacitracin per g. or cc											
	1			2			3			4		
	1	2	3	1	2	3	1	2	3	1	2	3
Blood	2.5	2.0	2.5	18.0	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Bile	3.65	1.8	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Cerebrospinal fluid	1.21	0.1	0	0	0	0	0	0	0	0	0	0
Urine	177.0	27.5	180.0	1.98	1.98	1.98	1.98	1.98	1.98	1.98	1.98	1.98
Kidney	3.0	3.1	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Lung	1.35	2.0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Pancreas	0.53	0.7	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Skeletal muscle	0.36	1.35	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Heart	0.39	0.9	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Spleen	0.27	0.72	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Liver	Trace	0	0	0	0	0	0	0	0	0	0	0
Brain	0.84	0.72	0.66	0.66	0.66	0.66	0.66	0.66	0.66	0.66	0.66	0.66
Bone marrow	0.82	3.6	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38
Skin	0.42	2.85	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
Large intestine	0.33	0.33	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34	0.34
Small intestine												

However, this barrier is overcome when the meninges are severely inflamed, as in suppurative meningitis, for then a significant level of bacitracin can be recovered in the cerebrospinal fluid.<sup>6</sup>

The high bacitracin content in the kidney is to be expected in view of the fact that this organ is active in the excretion of the drug. The concentration of bacitracin in the urine obviously depends to a considerable degree upon both the total intake and the total output of fluid. The output of urine could not be determined because the animals voided a variable amount during the course of the experiment and the urine was not collected. The concentrations noted in the table were those found in the urine present in the bladder at the time of death. The wide variation in these figures was not unexpected for it is similar to that found in patients following intramuscular injection. Although no albumin or casts were found in these urine specimens and there was no gross evidence of kidney damage at the time of autopsy, it is possible that there may have been some variation in renal blood flow or glomerular or tubular filtration to account for the wide variations in the urine concentrations. This might also explain the wide range in the blood levels.

Of interest and possibly of clinical importance is the relatively high bacitracin content in the lungs, the skin and the bone marrow. The content of the drug in the bone marrow is in contrast to that of penicillin, which according to the report of Struble and Bellows does not reach this tissue.<sup>4</sup> However, some doubt is cast upon this finding with regard to penicillin by the excellent response of acute osteomyelitis to treatment with this drug. This might be explained on the basis of increased permeability of the capillary walls in the early stages of an acute inflammation. The high figure for bile may be partially explained by the fact that a control specimen of bile showed an inhibitory effect against the growth of *Micrococcus flavus*. However the variation in the levels, with the peak average at two hours, suggests that bacitracin is ex-

<sup>6</sup>Teng, P., and Melency, F. L., accepted for publication in *Surgery*.

portance. Lack of rapid and complete equilibrium between the newly formed and injected acetylamino acids would also yield low radioactivity in the excreted product. Both acetate and alanine (pyruvate) carbons can be used in this acetylation.

*Summary.* Intravenously injected acetyl-

tryptophan and acetyl glycine are excreted in part by rats. Advantage was taken of this behavior to trap any radioactive acetylamino acids that are formed *in vivo*. The results show that natural amino acids can be acetylated *in vivo*.

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### Distribution of Bacitracin in the Body.\* (17394)

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Bacitracin is an antibiotic with a wide antibacterial spectrum. It was discovered in the Bacteriological Research Laboratory of the Department of Surgery of Columbia University. The active principle is produced by the Tracey I strain of *B. subtilis*.<sup>1</sup> Bacitracin has been used clinically in the treatment of many infections which had failed to respond to other antibiotic and chemotherapeutic agents.<sup>2</sup> Because of its clinical importance, it seemed to the authors worth while to determine the distribution of the drug in the different tissues and fluids of the experimental animal following intravenous administration.

*First series of experiments.* Eleven normal rabbits weighing from 2 to 4 kilos were selected. Bacitracin in a dosage of 3,000 units per kilo of body weight, dissolved in 3 or 5 cc of physiological saline, was injected slowly into the ear vein of each rabbit. One, 2 or 3 hours after the injection the animals were bled to death under nembutal anesthesia. The

whole organ or a portion of the tissue was then removed from the skin, heart, lung, liver, skeletal muscle, brain, bone marrow, pancreas, kidney, intestines and the spleen. Each kind of tissue was ground up in a mortar with an equivalent weight of physiological saline. It was then filtered through cotton. Each filtrate was then assayed for the concentration of bacitracin by the penicylinderplate method against *Micrococcus flavus*.<sup>3</sup> The cerebrospinal fluid, blood, bile and urine were also collected and their bacitracin content similarly determined. The results are presented in Table I.

*Discussion.* Although there are some wide variations, composite figures as expressed in averages give an approximate value for the various tissues. The figures seem to indicate that the antibiotic is widely distributed throughout the body, but its concentration is somewhat variable in different tissues. For example, the chorioid plexus apparently acts as a barrier to the passage of bacitracin. None or only a trace of bacitracin was found in the cerebrospinal fluid or in the brain tissues after intravenous or parenteral injection. This is also true of penicillin and streptomycin.<sup>4,5</sup>

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The bacitracin used was supplied by the Commercial Solvents Corporation of Terre Haute, Ind.

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3 Hott, D. A., Bennett, R. E., and Stanley, A. R., *Science*, 1947, **106**, 551.

4 Struble, G. G., and Bellows, J. G., *J.A.M.A.*, 1944, **125**, 685.

5 Buggs, C. W., Pilling, M. A., Bronstein, B., Hirschfeld, J. W., Worzniak, L., and Key, L. J., *J. Clin. Invest.*, 1946, **25**, 94.

small intestine, cerebrospinal fluid and brain.

In the chest and peritoneal exudates produced by aleuronat-starch injection, the titres

of bacitracin one hour after an intravenous administration approximated those in the blood.

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## Production of an Inactive Derivative of Purified Prothrombin by Means of Purified Thrombin.\* (17395)

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The blood clotting mechanism is of such complexity that the reactions which the several clotting factors can participate in may be followed with certainty only when these factors are isolated and obtained in purified form. With the use of such purified preparations, it has been shown that, depending upon the conditions of the experiment, thrombin may have entirely different effects on prothrombin. When small amounts of thrombin are added to prothrombin dissolved in 25% sodium citrate solution, the rate of activation of the prothrombin to thrombin is increased;<sup>1,2</sup> when thrombin and prothrombin react in physiological saline solution the prothrombin is changed to an inactive form in which it does not react to the addition of calcium, Ac-globulin and thromboplastin.<sup>3,4</sup> These effects are apparently produced consecutively when relatively large amounts of thrombin are used. There first appears a decrease in sensitivity to calcium, Ac-globulin and thromboplastin, then a reappearance of the original sensitivity. These experiments might be interpreted to mean that prothrombin is inactivated only to be changed back to its original active form

again. It appears more reasonable, however, that thrombin acts first to form a slightly modified prothrombin which is not reactive to thromboplastin, and that this modified prothrombin is then changed to another active form of prothrombin which yields thrombin. The sequence may be described by the following: Prothrombin (sensitive to calcium + Ac-globulin + thromboplastin)  $\rightarrow$  Prothrombin-derivative I (not sensitive to calcium + Ac-globulin + thromboplastin)  $\rightarrow$  Prothrombin-derivative II (sensitive to calcium + Ac-globulin + thromboplastin)  $\rightarrow$  Thrombin and possibly other reaction products. In this paper we shall show that the first prothrombin-derivative, a protein insensitive to calcium + Ac-globulin + thromboplastin, can be identified by electrophoresis.

*Experimental procedures.* A large homogeneous supply of prothrombin was obtained by pooling several preparations made as previously described.<sup>4-6</sup> Many experiments were performed but all of those described in this paper were performed on this pooled sample of prothrombin.

When dissolved in saline solution it retained at least 95% of its activity for 6 hours at room temperature. On electrophoresis the patterns (Fig. 1) showed that 95% of the total protein was present in one component in the descending boundary, while 87% was present in the ascending boundary. The impurities present in the prothrombin sample were thus shown to be sufficiently reduced to

\* Aided by a grant from the United States Public Health Service, National Institute of Health. Parke, Davis and Company supplied large quantities of plasma required for the preparation of purified prothrombin and thrombin.

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<sup>4</sup> Ware, A. G., and Seegers, W. H., *J. Biol. Chem.*, 1948, 174, 565.

<sup>5</sup> Seegers, W. H., Loomis, E. C., and Vandenbelt, J. M., *Arch. Biochem.*, 1945, 6, 85.

<sup>6</sup> Seegers, W. H., *J. Biol. Chem.*, 1949, 133, 163.

TABLE II.

Concentration of Bacitracin in the Chest and Peritoneal Fluids and in the Blood Serum of Rabbits after an Intravenous Injection of the Antibiotic.\* (3,000 units/kilo body wt).

Rabbit No.	Bacitracin levels in units/cc		
	Chest fluid	Peritoneal fluid	Blood serum
1	2.3	2.3	1.6
2	5.0		7.5
3	4.2		4.5
4		2.6	2.55
5	2.4		2.8
Avg	3.47	2.45	3.79

\* One hour after bacitracin injection and 25 hours after the intraperitoneal and intrathoracic injections of aleuronat-starch.

creted in the bile.

There was a moderate concentration of bacitracin in the wall of both the small and the large intestine. This suggests the possible value of the antibiotic in the treatment of intestinal infections, either bacterial or protozoan, provided that these infections are caused by organisms susceptible to bacitracin.

The liver, spleen, pancreas, heart and skeletal muscles all showed a moderate amount of the drug, which suggests that infections in these organs might be reached by the circulating drug.

*Second series of experiments.* In the second part of this study, 10 cc of aleuronat-starch, which contained 3% starch and 5% aleuronat, were injected into the pleural and into the peritoneal cavities of 5 normal rabbits 24 hours before the intravenous administration of bacitracin in the same dosages described above. These animals were sacrificed one hour after the injection of the antibiotic. The pleural or peritoneal exudate produced by the irritation of the aleuronat-starch was collected for a study of the bacitracin level by the penicylinder-plate method. The results are shown in Table II.

*Discussion.* These figures seem to indicate clearly that bacitracin diffuses into the exudate in the purulent response to a chemical irritant in experimental rabbits and presumably will diffuse into an exudate of infectious origin in man. In one rabbit, the bacitracin levels in the peritoneal and pleural fluid were higher

than those in the blood serum. In one the level in the chest fluid was lower, and in the others the titres were approximately the same as in the blood serum. Johnson, Anker and Meleney reported that subcutaneous bacitracin could save experimental mice following the intraperitoneal injection of 10,000-100,000 minimum lethal doses of hemolytic streptococci.<sup>1</sup>

In 3 clinical cases of ascites, from 1 to 6 hours after a single intramuscular injection of 49,000 units of bacitracin, which was approximately 1,000 units per kilo of body weight, Michie found that the concentration of the drug varied from 0.0018 to 0.128 unit per cc of ascitic fluid. In the pleural fluid of one patient he demonstrated a level of 0.5 unit per cc 4 hours after an intramuscular injection of 40,000 units of bacitracin.<sup>7</sup>

The fluid collected from the above patients was of a transudative nature. In the case of exudates, a higher bacitracin content might well be expected. Whenever the permeability of blood vessels is increased by an inflammatory process, it is easier for bacitracin to pass through the capillary bed and enter the inflamed tissues.

During the early commercial production of bacitracin, certain lots showed a disturbing degree of kidney irritation which interfered with systemic administration, but since July of 1948 our experience has shown that a product which meets the Food and Drug Administration's specification for toxicity of an LD50 of 500 units for 20-g mice, produces only a minimal and transient degree of kidney irritation. The lot used in these experiments, namely, No. 480408, met that specification and has been used without difficulty by intramuscular injection in a large number of human infections.

*Summary.* One, 2 or 3 hours after a large single intravenous injection of bacitracin, the concentration of the drug in the different tissues of rabbits was found in decreasing order as follows, subject to some individual variation: urine, kidney, blood, bile, lung, bone marrow, skin, large intestine, pancreas, heart muscle, skeletal muscle, liver, spleen,

<sup>7</sup> Michie, A., work in progress.

due to the action of small amounts of thrombin, formed spontaneously from the prothrombin. It was not appreciated at the time that the product formed from the prothrombin was the principal impurity of the preparation. The thrombin produced a derivative of prothrombin which was without typical activity. It is likely that the presence of this inactive prothrombin derivative is responsible for the polydisperse appearance of the prothrombin preparations examined in the ultracentrifuge.<sup>10</sup>

*Summary.* When purified prothrombin was

allowed to react with a small amount of purified thrombin there was loss in prothrombin activity. Comparison of the electrophoretic patterns of the prothrombin before and after alteration with thrombin showed that protein disappeared from the curve representing prothrombin and appeared as a new component with a lower electrophoretic mobility than that of prothrombin.

<sup>10</sup> Seegers, W. H., and Ware, A. G., *Fed. Proc.*, 1948, 7, 186.

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## Acetylcholine and Blood Sugar. (17396)

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Intravenous injection of acetylcholine (100-500  $\mu$ /kg, i.v.) produces hyperglycemia (25-60%) in rabbits. High doses of acetylcholine (500-1000  $\mu$ /kg, i.v.) induce convulsions and a marked increase in blood sugar, up to 200%.

The mechanisms of the hyperglycemia provoked by acetylcholine have been investigated in a series of experiments. The observations may be summarized as follows:

1. Atropine (0.25 - 1 mg/kg, i.v.) does not abolish the acetylcholine hyperglycemia

2. The adrenolytic drug SY 28 ( $\alpha$ -naphthylmethyl-ethyl- $\beta$ -bromo-ethylamine HBr), in doses of 1 to 3 mg/kg, does not abolish the hyperglycemia induced by acetylcholine or epinephrine.

3. The anticholinesterase drug: dimethylcarbamate of hydroxyphenyl-benzyl-trimethylammonium (Nu-683) (0.1 - 0.3 mg/kg i.v.) sensitizes the animal very markedly to the hyperglycemic actions of acetylcholine.

4. Tetraethylammonium (20 mg/kg i.v.) protects against acetylcholine-hyperglycemia, if doses of acetylcholine which do not induce convulsions are injected.

5. After removal of both adrenal glands and treatment with desoxycorticosterone-acetate, rabbits do not present hyperglycemia after doses of acetylcholine which do not in-

duce convulsions. Higher doses of acetylcholine inducing convulsions still provoke hyperglycemia.

6. Nembutal (Sodium-ethyl-(1-methyl-butyl) barbiturate) anesthesia prevents acetylcholine-hyperglycemia.

7. Control experiments show that no changes in blood sugar occur after injections of the same doses of atropine, tetraethylammonium SY 28 or Nu-683.

8. High doses of Nu-683 (0.5 mg/kg) induce hyperglycemia, which is also prevented by tetraethylammonium.

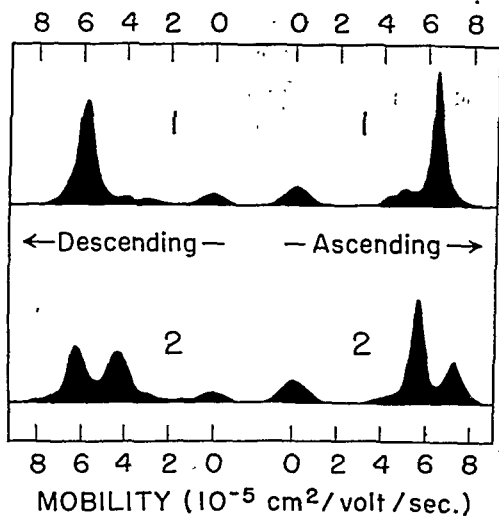
*Conclusions.* In rabbits, doses of acetylcholine which do not provoke convulsions induce hyperglycemia.

This increase in blood sugar is prevented by the synaptic blocking agent tetraethylammonium, by nembutal and by removal of both adrenal glands.

The acetylcholine-hyperglycemia thus is induced by adrenal synaptic stimulation, which increases epinephrine output.

The acetylcholine and epinephrine hyperglycemia are not abolished by an adrenolytic agent (SY 28). The hyperglycemia induced by high doses of an anticholinesterase drug (Nu-683) is also prevented by tetraethylammonium.

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## 1. Prothrombin control

## 2. Prothrombin partially inactivated with Thrombin

FIG. 1.

The electrophoretic boundary patterns of purified bovine prothrombin and of the same prothrombin after partial inactivation with purified thrombin, in barbital buffer, pH 8.5, ionic strength 0.1, and after 150 min. electrophoresis.

permit a study of the effect of thrombin on the boundary patterns and electrophoretic mobility of prothrombin.

The purified thrombin was prepared by dissolving sufficient purified prothrombin in a 25% solution of sodium citrate to make a 1% solution, adding a small amount of 3-methyl-4, 6, 4'-triaminodiphenyl sulfone and allowing the solution to stand at room temperature for 24 hours. The addition of the sulfone increases the yield of thrombin to a maximum. To separate the formed thrombin from the other substances present it was precipitated by the addition of ammonium sulfate, redissolved in water, and dialyzed free of salt. This method of preparing thrombin will be described in detail and presented for later publication. The important feature of this method is that thrombin is obtained from prothrombin without the use of calcium, Ac-globulin or thromboplastin and is thus free of these substances and other impurities present in even the best Ac-globulin and thromboplastin preparations now available. Throm-

bin prepared in this way is of higher purity than any previously described, and is of sufficient activity that in the amounts used in our reaction mixtures it could not be detected electrophoretically in the presence of the prothrombin.

Reaction mixtures were prepared from these purified preparations by dissolving in physiological saline sufficient prothrombin to give a concentration of 7,700 units per cc of solution and adding sufficient dry thrombin to bring its concentration to 100 units. The reaction mixture was allowed to stand for 6 hours at room temperature, when samples were removed for measurement of prothrombin activity, by the modified two-stage method<sup>7,8</sup> of analysis and the remainder of the reaction mixture was frozen, dried from the frozen state, dissolved in barbital buffer at pH 8.4 and 0.1 ionic strength and subjected to electrophoresis. The prothrombin activity decreased during the period of incubation to 62% of the original value. Comparison of the electrophoretic patterns of the prothrombin before and after incubation with thrombin (Fig. 1) reveals that the major component of the original sample was reduced in quantity from 87% to 32% in the ascending boundary and from 94% to 53% in the descending boundary. This protein which has disappeared from the major boundary reappeared in a second boundary which represented 64% of the total protein in the ascending boundary and 43% in the descending boundary. The decrease in prothrombin activity as measured by the two-stage method of analysis was in rough agreement with the shift of protein from the main electrophoretic component to the second component. The inactive derivative of prothrombin is characterized by an electrophoretic mobility of 5.60 (ascending) and 4.21 (descending)  $\times 10^5$  cm<sup>2</sup>/volt/sec.

One of the principal difficulties encountered in early work<sup>9</sup> on the purification of prothrombin was the loss of prothrombin activity

<sup>7</sup> Ware, A. G., and Seegers, W. H., *Am. J. Clin. Path.*, 1949, **19**, 471.

<sup>8</sup> Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

<sup>9</sup> Ware, A. G., Guest, M. M., and Seegers, W. H., *Am. J. Physiol.*, 1947, **150**, 58.

TABLE I.  
Thyroid Weight of Adult Hens Fed Diets Containing Large Amounts of Inorganic Iodide, and  
Thyroid Weight of Their Progeny on Day of Hatch.

Diet of hens	Amt thyroxine added to diet, (mg/lb)	Amt inorganic I, added to diet, (mg/lb)	Mean thyroid wt* of chicks, (mg $\pm$ S.E.)	Mean thyroid wt† of hens, (mg $\pm$ S.E.)
Control	—	—	3.0 0.06	98 16.9
.04% STP‡	6	—	7.2 0.22	22 0.7
.001% KI	—	4	7.0 0.15	149 45.9
.002% "	—	8	8.1 0.12	162 16.9
.005% "	—	16	8.5 0.33	143 43.9
.009% "	—	32	8.4 0.32	352 30.0
.018% "	—	64	12.6 1.18	330 63.9

\* Avg of thyroids from 10 day-old chicks.

† Avg of paired thyroids from 7 hens except in groups fed 32 and 64 mg I per lb of feed, which included 6 and 5 paired thyroids, respectively.

‡ STP, trade name Protamone, furnished by Cerophyl Laboratories, Kansas City, Mo.

supplemented with varying amounts of iodide (as KI), or STP. It is necessary to emphasize the excessive nature of the iodide levels. To meet the recommendation of the National Research Council, practical poultry diets should contain 0.5 mg iodine per pound. The diets employed in the present study contained 8 to 128 times this amount. The results are presented in Table I. It will be observed that the feeding of KI induced thyroid enlargement in the adult hens, confirming the goiterogenic action of excessive amounts of inorganic iodide which had been previously demonstrated in the chick embryo. As expected, STP caused thyroid involution in the adults, indicating that the thyroxine intake was sufficient to inhibit thyrotropin secretion.

The offspring of KI-fed hens also showed marked thyroid enlargement. It is of particular interest that hens fed approximately the same amount of iodide as was supplied by STP, produced chicks with thyroid enlargement comparable to that in the offspring of the STP-fed hens. Apparently the thyroid enlargement found in chicks from STP-fed hens is associated with the iodide rather than with the thyroxine in STP. This would explain why the feeding of desiccated thyroid,<sup>7</sup> or STP<sup>1,2</sup> to hens, or the injection of crystalline thyroxine into hens,<sup>3</sup> fails to decrease thyroid size in their chicks. Apparently any thyroxine naturally deposited in the egg is metabolized or inactivated by the time the chick

thyroid becomes functional (about the 14th day of incubation) and, therefore, fails to modify thyroid size. It should be noted, however, that if STP<sup>6</sup> or thyroxine<sup>3,6</sup> is injected directly into eggs during the first week of incubation, chick thyroid size at time of hatch is significantly reduced.

The hypothyroid symptoms of offspring of hens fed inorganic iodide are essentially the same as those in chicks from eggs injected with thiourea,<sup>12</sup> or KI<sup>9</sup>; in chicks from dams fed STP,<sup>12</sup> and in squabs from iodine-deficient dams.<sup>13</sup> The delay in hatching caused by feeding iodide to hens may explain the findings of Wilgus, *et al.*<sup>14</sup> that hatchability was unfavorably affected by amounts of iodine in the hen's diet in excess of 23 mg/lb. In the present study no decrease in hatchability was noted if the eggs were left in the incubator an extra day to allow for the delayed-hatching effect of the iodide supplement.

Further evidence of the like effects of feeding KI and STP to the hens on the thyroid glands of newly hatched chicks has been obtained from histological sections. (Fig. 1.) The thyroid glands of chicks whose dams were

<sup>12</sup> Grossowicz, N., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 151.

<sup>13</sup> Hollander, W. F., and Riddle, O., *Poultry Sci.*, 1946, **27**, 20.

<sup>14</sup> Wilgus, H. S., Gassner, F. N., Patton, A. R., and Harshfield, G. S., *Poultry Sci.*, 1948, **27**, 686. (abstract).



## Goiterogenic Action of Iodide and the Etiology of Goiters in Chicks from Thyroprotein-fed Hens. (17397)

ROBERT S. WHEELER AND EDMUND HOFFMANN. (Introduced by M. Juhn.)

From the Department of Poultry Husbandry, University of Georgia, Athens, Ga.

It has been shown recently that the feeding of synthetic thyroprotein (STP) to hens increases the time required to hatch their eggs and results in the presence of goiters in the chicks hatched from these eggs.<sup>1,2</sup> These chicks are apparently hypothyroid as shown by their lowered oxygen consumption rate.<sup>3</sup> These results are contradictory because STP fed directly to adults and growing stock<sup>4,5</sup> results in an elevated metabolic rate and in thyroid involution. Injection of STP into fertile eggs also results in thyroid involution.<sup>6</sup> In the present communication, a mechanism involved in this paradox is established. It had been postulated that maternal thyroxine deposited in the egg regulates the thyroid function of the embryonic chick. Thus the goiters in chicks from STP-fed dams were believed due to reduced maternal thyroid activity with attending failure of the thyroidal substance in STP to be deposited within the egg. This hypothesis proved untenable, however, when the feeding of desiccated thyroid to hens failed to alter incubation time of their eggs or thyroid size of their chicks.<sup>7</sup>

A new hypothesis based on the relatively high inorganic iodide content of STP was developed following the report by Wolff and Chaikoff that the administration of large amounts of inorganic iodide inhibited thyroxine synthesis in the thyroid gland of the nor-

mal rat.<sup>8</sup> In these experiments thyroid inhibition was of short duration and it is not surprising that thyroid enlargement did not occur. However, it might be expected that prolonged inhibition of the thyroid by any agent would result in a compensatory enlargement. As a result of our efforts to test this thesis, it has been demonstrated that inorganic iodide can act as a goiterogenic agent.<sup>9</sup> When large amounts of inorganic iodide were injected into the white of fertile eggs on the 16th day of incubation, the chicks exhibited marked thyroid enlargement at the time of hatch. Moreover, these goiterous chicks exhibited typical hypothyroid symptoms: delayed hatching and delayed closure of the umbilicus.

To establish inorganic iodide as the cause of goiters in chicks from STP-fed dams, it was necessary to demonstrate that the inorganic iodide content of the hen's diet could affect the thyroid size of her chicks. This might be expected since the iodide content of the hen's diet determines to a large extent the iodine content of her eggs.<sup>10</sup> In the commercial manufacture of STP, casein is incubated with elemental iodine and the resulting product contains 1.5-2.0% inorganic iodine as iodide as well as 3.0% crystalline thyroxine.<sup>11</sup> Hence, it seems logical that this inorganic iodide in STP might increase the iodide content of the egg sufficiently to inhibit thyroxine synthesis in the embryonic chick thyroid and produce a compensatory thyroid enlargement.

**Experimental.** To test this hypothesis, groups of 12 hens were fed a conventional diet

<sup>1</sup> Wheeler, R. S., and Hoffmann, E., *Endocrinology*, 1948, **42**, 326.

<sup>2</sup> Wheeler, R. S., and Hoffmann, E., *Endocrinology*, 1948, **43**, 430.

<sup>3</sup> McCartney, M. G., and Shaffner, C., *Poultry Sci.*, 1949, **23**, 223.

<sup>4</sup> Wheeler, R. S., Hoffmann, E., and Graham, C. L., *Poultry Sci.*, 1948, **27**, 102.

<sup>5</sup> Wheeler, R. S., and Hoffmann, E., *Poultry Sci.*, 1948, **27**, 509.

<sup>6</sup> Booker, E. E., and Sturkie, P. D., *Poultry Sci.*, 1949, **28**, 147.

<sup>7</sup> Brooke, J. F., and Wheeler, R. S., unpublished data.

<sup>8</sup> Wolff, J., and Chaikoff, I. L., *Endocrinology*, 1948, **42**, 468.

<sup>9</sup> Wheeler, R. S., and Hoffmann, E., *Endocrinology*, 1949, **45**, 208.

<sup>10</sup> Wilder, O. H. M., Bethke, R. M., and Record, P. R., *J. Nutrition*, 1933, **6**, 407.

<sup>11</sup> Graham, W. R., Jr., personal communication. Cerophyl Laboratories, Kansas City, Missouri.

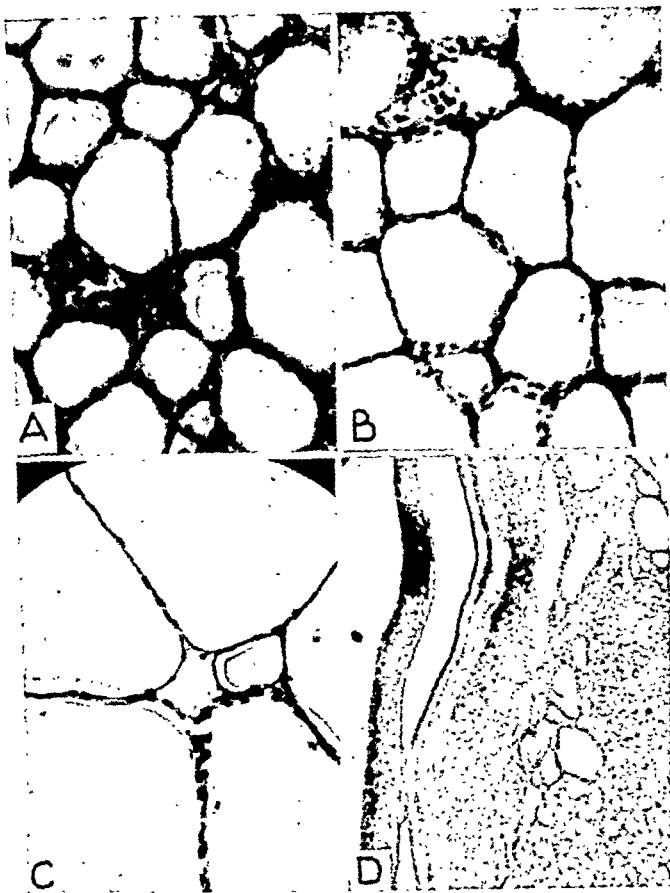


FIG. 2.

Histological sections of the thyroid gland of laying hens. A. Thyroid of normal hen. B. Thyroid of STP-fed hen. C. Thyroid of KI-fed hen. D. Low power field of the "normal" thyroid tissue and pseudo-encapsulation found in the nodular, colloid goiter observed in three out of fourteen hens when the higher dosages of KI were fed. Note the transitional tissue progressing from right to left. The condensed tissue to the extreme left forms the border of a large colloid nodule or cyst. A, B, C, 225 X; D, 75 X.

smallest was 35 x 30 mm in the longest and shortest dimension as compared to 7 x 3 mm in size for glands of normal birds. Some of the nodules (cysts) observed in these goiters were as large as 10 mm in diameter.

**Discussion.** It is difficult to integrate the present finding of a goiterogenic effect of iodide in the normal animal with the iodine-thyroid literature. Morton, Chaikoff and Rosenfeld showed<sup>16</sup> that thyroxine synthesis by thyroid slices *in vitro* was inhibited by the presence of an excess of iodide ions. In later work, Wolff and Chaikoff<sup>17</sup> obtained an inhi-

bition of thyroxine synthesis *in vivo* which could be prolonged by nephrectomy. They suggest that the effect of large doses of inorganic iodide on a hyperactive gland (Graves' disease) is to reduce thyroxine secretion and thus ameliorate the symptoms of hyperthyroidism.<sup>17</sup> One might guess from this work that continued suppression of the thyroxine secretion of the normal gland would lead to

<sup>16</sup> Morton, M. L., Chaikoff, I. L., and Rosenfeld, S., *J. Biol. Chem.*, 1944, 154, 381.

<sup>17</sup> Wolff, J., and Chaikoff, I. L., *J. Biol. Chem.*, 1945, 172, 855.

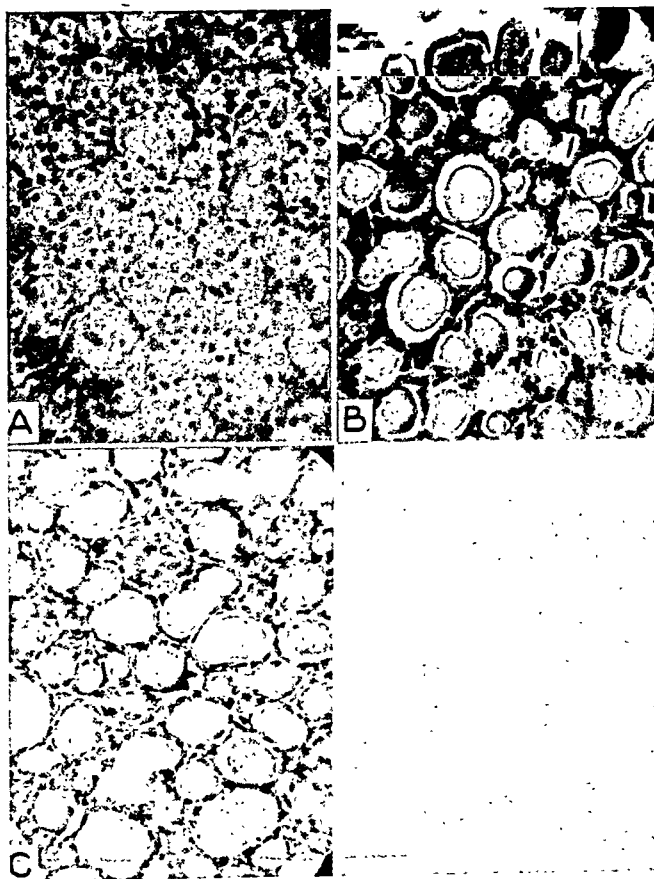


Fig. 1.

Histological sections of the thyroid gland of day-old chicks 225 X. A. Normal chick thyroid. B. Thyroid of chick from STP-fed hen. C. Thyroid of chick from KI-fed hen.

fed either KI or STP are indistinguishable; the many follicles are distended with colloid and the epithelial cells are flattened. In the normal gland very few follicles are seen and the epithelial cells are cuboidal.

Histological sections of the thyroid glands of hens fed KI are of interest because of marked colloid storage. (Fig. 2.) In most cases the follicles were so large that it was not possible to photograph a sufficient area to convey the true picture. It would appear that the generally accepted concept of Marine<sup>15</sup> that colloid goiter is the result of involution of a previously hyperplastic gland, cannot explain the tremendous enlargement

observed in the present study after only one month of treatment. In 3 cases where hens received the 2 highest dosages of KI, goiters with colloid nodules and cystic degeneration were observed. A line drawing of one of these glands is presented in Fig. 3. Apparently the follicles become confluent and form large colloid vesicles surrounded by a pseudo encapsulation. There was no evidence of neoplasm. However, the trabeculae forming the boundaries of the vesicles contained some thyroid tissue which appears to be normal and some thyroid tissue showing only slightly increased colloid storage. The weights of these glands were not included in the data because of their extreme deviation from the mean. The largest gland was 50 x 35 mm and the

<sup>15</sup> Marine, D., *Medicine*, 1924, 3, 453.

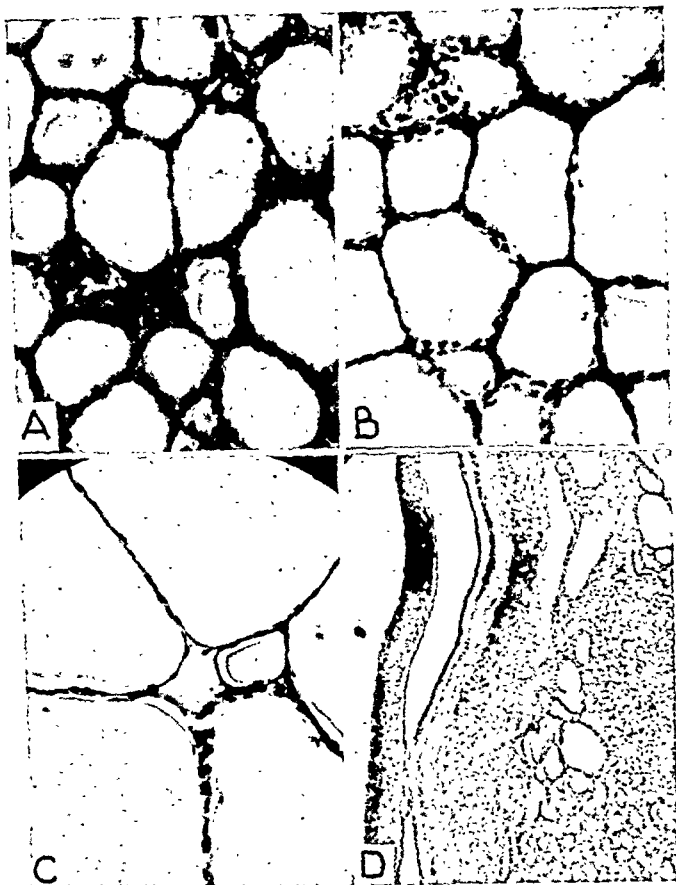


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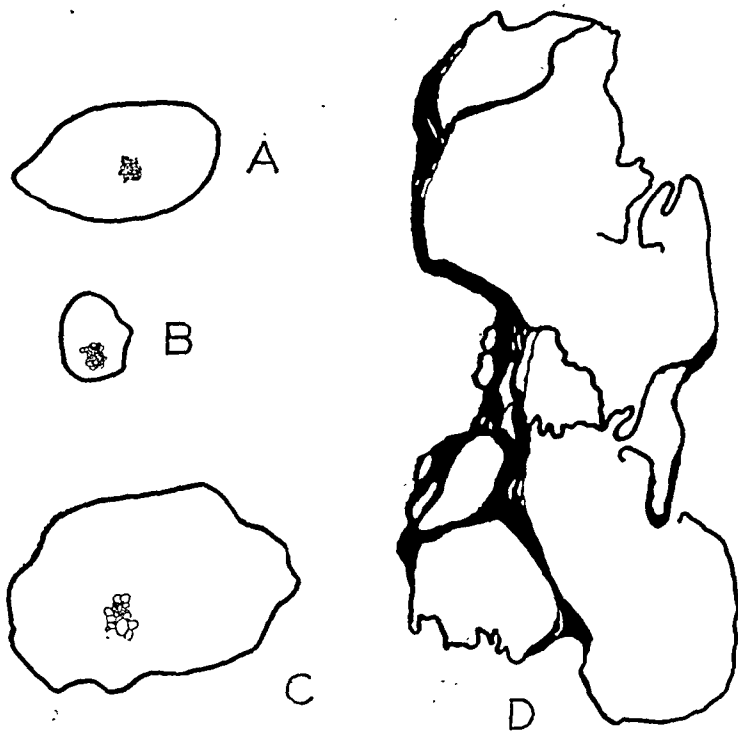


FIG. 3.

Line drawing of median frontal sections of thyroid from: A. Normal hen. B. STP-fed hen. C. KI-fed hen. D. KI-fed hen showing nodular colloid goiter with cystic degeneration. Note the relative follicle size in A, B, C. All figures 4.3 X.

subsequent enlargement of the thyroid via increased thyrotropin from the pituitary.

In the absence of histological evidence this might explain the thyroid enlargement resulting from administration of large doses of iodide. But Rawson *et al.*<sup>18</sup> have differentiated between the antithyroid action of iodide and thiouracil on a hyperplastic gland. Whereas thiouracil produces a columnar epithelium in a gland devoid of colloid, iodide produces an involution of epithelial cells and the storage of colloid. These effects of iodide have been recently confirmed for normal animals by Lesser, Winzler and Michaelson,<sup>19</sup> who also noted that rats fed KI but exposed to cold had more normal appearing glands than

treated rats held at room temperature. Thus, in this instance, increased thyrotropin partly reduced the colloid storing effect of iodide. In the light of these findings, it is doubtful if an increase in thyrotropin secretion can adequately explain the goiterogenic effects of iodide, but no alternative hypothesis can be proposed at this time.

**Summary.** The goiterogenic action of increased iodide intake is demonstrated both in the normal fowl, following continuous treatment, as well as in the developing chick embryo. The goiters in chicks presumably result from large amounts of inorganic iodide deposited in the egg by the hen and are comparable in weight and histology to those observed in chicks from dams fed synthetic thyroprotein. It is suggested that the goiters observed in chicks from dams fed synthetic thyroprotein are caused by the iodide present in thyroprotein.

<sup>18</sup> Rawson, R. W., Moore, F. D., Peacock, W., Means, J. H., Cope, O., and Riddell, C. B., *J. Clin. Invest.*, 1945, **24**, 869.

<sup>19</sup> Lesser A. J., Winzler, R. J., and Michaelson, J. B., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 571.

## A Method for Experimental Production of Gradual Occlusion of the Portal Vein.\* (17398)

PETER W. STONE AND RALPH A. MURPHY, JR.<sup>†</sup> (Introduced by J. V. Warren.)

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A sudden complete occlusion of the portal vein is invariably fatal. However, a gradual occlusion of the portal vein in the dog and other laboratory animals is compatible with life, as was demonstrated first by Oré<sup>1</sup> and later by Bernard,<sup>2</sup> Tilmann,<sup>3</sup> Ito and Omi,<sup>4</sup> Neuhof,<sup>5</sup> Boyce *et al.*,<sup>6</sup> Dragstedt,<sup>7</sup> and Brunschwig *et al.*<sup>8</sup> Survival is dependent upon the development of collateral venous channels in the gastrohepatic mesentery, and anastomoses with the esophageal, caval, renal, and rectal veins.

Gradual occlusion of the portal vein has been used by Dragstedt<sup>7</sup> as a substitute for Eck fistula in physiologic studies of the liver and the abdominal organs draining into the portal system. It has also been studied experimentally by Brunschwig *et al.*<sup>8</sup> with a more clinical application in mind. The proximity of the portal vein to the pancreas, and its occasional invasion by pancreatic carcinoma in man, led Brunschwig and his co-workers to investigate methods for the production of adequate collateral channels, thereby enabling excision of this vessel. Menon<sup>9</sup> and Kershner

*et al.*<sup>10</sup> attempted to produce portal hypertension in the laboratory animal by gradual occlusion of the portal vein. Consistent increases in portal venous pressure with ascites, esophageal varices, and splenomegaly were not obtained.

All of the methods reported for gradual occlusion of the portal vein were ligature technics with various modifications. Each of the technics required at least a 2-stage operative procedure, with the exception of that described by Brunschwig *et al.*<sup>8</sup> These workers were able to occlude the portal vein successfully by means of a linen thread looped around the vein, the ends of which were brought out through the operative wound and tied over the back of the animal. These ends were pulled upon slightly each post-operative day until the intact loop was pulled out, denoting that the loop had passed through the portal vein. Although gradual occlusion of the portal vein was accomplished successfully by this method, the incidence of fatal peritonitis was high.

The present study is a report of the use of irritative cellophane (Polythene<sup>‡</sup>) and tantalum to produce gradual portal vein occlusion. It is believed that this method possesses certain advantages over the ligature technics formerly used. It eliminates the repeated operative procedures necessitated by ligation in stages, and also obviates the substantial incidence of peritonitis associated with ligature transection of the portal vein.

*Method.* Healthy adult mongrel dogs were used. Following intravenous sodium pento-

\* This project received financial support from the Medical Research and Development Board, Office of the Surgeon General, U. S. Army.

<sup>†</sup> Life Insurance Medical Student Research Fellow.

<sup>1</sup> Oré, *Compt. rend. Acad. Royal d. sc.*, 1856, **43**, 463 (cited by Brunschwig *et al.*<sup>8</sup>).

<sup>2</sup> Bernard, C., J. B. Baillière et fils, Paris, 1877, p. 316 (cited by Brunschwig *et al.*<sup>8</sup>).

<sup>3</sup> Tilmann, H., *Deutsche med. Wchnschr.*, 1899, **25**, 284.

<sup>4</sup> Ito, H., and Omi, K., *Deutsche Z. f. Chir.*, 1902, **62**, 141 (cited by Brunschwig *et al.*<sup>8</sup>).

<sup>5</sup> Neuhof, H., *Surg., Gynec. and Obst.*, 1913, **16**, 481.

<sup>6</sup> Boyce, F. F., Lampert, R., and McFetridge, E. M., *J. Lab. and Clin. Med.*, 1935, **20**, 935.

<sup>7</sup> Dragstedt, L. R., *Science*, 1931, **73**, 315.

<sup>8</sup> Brunschwig, A., Bigelow, R., and Nichols, S., *Surgery*, 1945, **17**, 781.

<sup>9</sup> Menon, T. B., *J. Path. and Bact.*, 1938, **46**, 357.

<sup>10</sup> Kershner, D., Hooton, T. C., and Shearer, E. M., *Arch. Surg.*, 1946, **53**, 425.

<sup>‡</sup> Polythene (DuPont), type NV-7-14 with diethyl phosphate, 1.5 mils in thickness, furnished by The Cellophane Division, E. I. DuPont de Nemours and Company, Wilmington, Del.

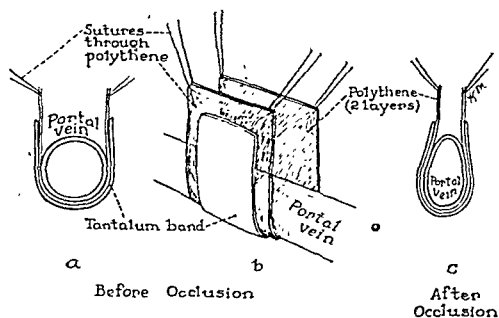


FIG. 1.

Schematic drawing illustrating application of Polythene and tantalum band to the portal vein.

barbital anesthesia, the abdomen was opened through a high right upper rectus muscle-splitting incision. The portal vein was mobilized and a double thickness cuff of Polythene, 1 to 2 cm in length, was wrapped about the vein at the porta hepatis. The Polythene casing was anchored by 2 interrupted No. 00000 arterial silk or cotton sutures. A narrow tantalum band was then placed about the Polythene and tightened so as to decrease the transverse diameter of the portal vein to approximately one-half to two-thirds of the original size (Fig. 1). A cuff of Polythene protruded from beneath the band at either end, and the tantalum did not contact the vein at any point.

After a period of 5 to 10 minutes the small bowel was examined for evidence of dangerously increased venous congestion. If marked venous congestion with cyanosis of the bowel was present, the tantalum band was loosened slightly. The abdominal wall was closed in layers only after the bowel was seen to be normal. A slight distention of the intestinal veins, with approximately a doubling of venous pressure, was usually present. Venous pressures in the portal bed were measured before and after placing of the tantalum band by means of the Burch phlebomanometer or by a spinal fluid manometer.

The animals were either re-explored or sacrificed 3 to 60 days later and the portal vein removed for gross and microscopic examination.

**Results.** The portal vein in 28 dogs was wrapped with Polythene and partially occluded with tantalum. One dog died within

3 hours of the time of operation, and 3 dogs expired within 12 hours. Postmortem examination revealed the characteristic findings of sudden acute portal occlusion. The mesenteric vessels were engorged, the spleen congested, and there were hemorrhagic areas in the mesentery and intestinal wall.

The remaining 24 dogs showed no immediate ill effects from the procedure. The portal vein was excised from 16 of these dogs without a fatality, at intervals ranging from 5 to 60 days (Fig. 2). At the time of the second operation, extensive collateral venous channels were present in the gastrohepatic mesentery. In addition, an increased tendency for the animal to go into shock was noted at the second operation, and was combatted by the use of intravenous fluids and cardiac stimulants. This was an important finding and deserves further study. Two dogs expired following portal vein excision on the fifth postoperative day. The remaining 6 dogs were sacrificed at varying intervals, with the collateral circulation being extensively studied.

Upon gross examination, the wall of the portal vein was thickened, the extent of thickening being dependent upon the length

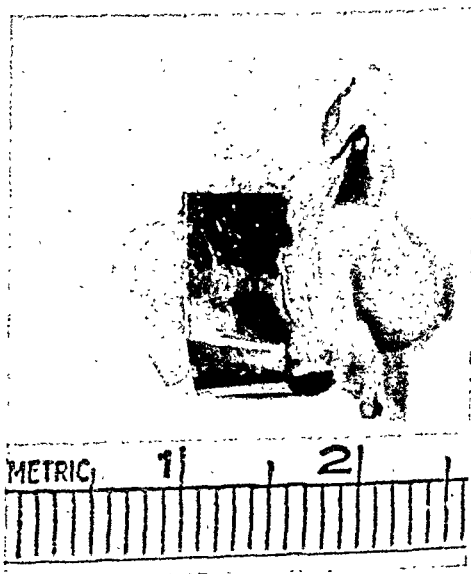


FIG. 2.

Photograph of excised portal vein with the Polythene and tantalum band in place.



FIG. 3.

a. Photograph of excised portal vein segment showing the reaction caused by the application of Polythene 60 days before. Note the absence of lumen and the marked thickening of the vessel walls.

b. Normal portal vein segment.

of time during which the vessel had been in contact with the Polythene. The lumen was reduced in all animals and by 30 days after wrapping only a small aperture remained. The lumen was completely obliterated in 4 dogs at 40 to 60 days. Surrounding the vein there was a moderately thick, brown gelatinous pseudomembrane (Fig. 3).

Microscopic examination of the portal vein from animals dying within 12 hours showed a fibrinous reaction with occasional lymphocytic infiltration in the tissues immediately adjacent to the portal vein. At 5 days, changes consisted of edema of the media and adventitia, round cell infiltration, and beginning fibroplasia; in addition, a fibrinous deposition in the tissues adjacent to the vein was noted. Localized hemorrhage into the wall of the vein was occasionally seen. At 10 to 15 days the acute reaction was less evident and the significant finding was medial, adventitial, and periportal fibrosis. At 18 to 20 days there was increased fibrosis and chronic inflammatory response in the outer half of the vein wall and periportal tissues. After a period of 30 to 60 days, intimal thickening with marked fibrosis of the media and adventitia was present (Fig. 4). Apparently the degree of fibroplasia was dependent upon the duration of Polythene application. It was also noted that the chronic inflammatory response was significantly decreased after 30 days.

✓ One of the original objectives of the study

was to attempt to produce portal hypertension by this method. However, at the time of re-exploration there was no elevation of venous pressure in the intestinal veins. Extensive collateral channels joined the renal, rectal, and caval veins, and were present in the gastrohepatic omentum. In some instances gastrohepatic collateral vessels were larger than the portal vein prior to Polythene and tantalum wrapping. The number and size of the collateral vessels was in direct proportion to the duration of portal occlusion. Injection studies of the venous collaterals, using liquid latex and Hill's media, revealed no instance of a collateral vessel passing directly into the liver. Significant anastomoses with esophageal veins were not demonstrated. This is of interest when compared with the usual collateral channels found in man following blockage of the portal vein.

*Discussion.* Irritative cellophane has been used in the treatment of aneurysms,<sup>11,12,13</sup> the experimental production of aortic stenosis and obliteration,<sup>14,15</sup> and in attempts to obliterate a patent ductus arteriosus.<sup>16</sup> The dicetyl phosphate present in the Polythene is responsible for the irritative properties of the material,<sup>17</sup> and differentiates this cellophane from the non-irritating type which does not induce fibroplasia and which has been used in certain orthopedic procedures.<sup>18,19</sup> We have not encountered any reports in the literature regarding the use of irritative cellophane in occlusion of major veins. It has

<sup>11</sup> Harrison, P. W., and Chandy, J., *Ann. Surg.*, 1943, **118**, 478.

<sup>12</sup> Poppe, J. K., and De Oliveira, H. R., *J. Thoracic Surg.*, 1946, **15**, 186.

<sup>13</sup> DeTakats, G., and Reynolds, J. T., *Surgery*, 1947, **21**, 443.

<sup>14</sup> Pearse, H. E., *Ann. Surg.*, 1940, **112**, 923.

<sup>15</sup> Cooper, F. W., Jr., Robertson, R. L., Shea, P. C., Jr., and Dennis, E. W., *Surgery*, 1949, **25**, 184.

<sup>16</sup> Harper, F. R., and Robinson, M. E., *Am. J. Surg.*, 1944, **64**, 294.

<sup>17</sup> Yeager, G. A., and Cowley, R. A., *Ann. Surg.*, 1948, **128**, 509.

<sup>18</sup> Wheeldon, T., *J. Bone and Joint Surg.*, 1939, **21**, 393.

<sup>19</sup> McKeever, D. C., *J. Bone and Joint Surg.*, 1943, **25**, 576.





FIG. 4.

a. Photomicrograph of a segment of portal vein 30 days after the application of Polythene and tantalum ( $\times 25$ ). Note the marked fibrosis and chronic inflammatory response in the media and adventitia.

b. Photomicrograph of a normal portal vein segment ( $\times 25$ ).

been stated that the aorta of the dog may be occluded with Polythene and tantalum in 12 weeks.<sup>15</sup> The interval required for total occlusion of the portal vein was approximately 6 to 8 weeks and the tissue response of the vein was similar to that described for the aorta.

The 4 dogs which died within 12 hours of operation illustrate the difficulty in securing maximal immediate constriction without occluding the vein to a point incompatible with life.

The shortest time interval compatible with survival between partial ligature occlusion of the portal vein and excision or complete ligation of this vessel has been reported as 10 days.<sup>6,8</sup> Four of 6 dogs in this series which were subjected to excision 5 days after partial occlusion survived. This suggests the development of a more extensive collateral venous network with the present method of occlusion, and clinically may be important in preparing for elective ligation of the portal vein.

It is believed that the gradual fibroplastic obliteration of the portal vein produced by the irritating cellophane (Polythene) more closely approximates the gradual reduction of portal blood flow as seen in certain disease states, *e.g.* the cirrhotic diseases, and will facilitate the study of these diseases.

**Conclusions.** 1. The fibroplastic response in the outer half of the vein wall and in the periportal tissues is similar to the reaction seen in the aorta following application of Polythene and tantalum.

2. At least 6 to 8 weeks are required for complete occlusion of the portal vein by Polythene-induced fibroplasia.

3. The gradual occlusion of the portal vein produced by irritative Polythene may be valuable in preparing for elective ligation of the portal vein and in the experimental study of decreased portal blood flow.

**Summary.** A one stage procedure for gradual occlusion of the portal vein using an irritative type of cellophane (Polythene) and tantalum is described.

The portal vein in 28 dogs was wrapped with Polythene and partially occluded with a tantalum band. The veins were removed for gross and microscopic examination 3 to 60 days later. Extensive venous collateral channels developed in the gastrohepatic mesentery and along the posterior peritoneum,

passing into the renal, rectal, and caval veins. In 4 of 6 dogs venous anastomoses were adequate to sustain life following excision of the portal vein 5 days after partial portal occlusion.

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### Neutralization of Three Immunological Types of Poliomyelitis Virus by Human Gamma Globulin.\* (17399)

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Recent studies of antigenic groups of poliomyelitis virus have shown the existence of at least 3 distinct types.<sup>1,2</sup> Two of these are widespread in their distribution, but the third is represented thus far by only one strain (Leon) isolated in Los Angeles, California in 1937.<sup>3</sup> In an attempt to determine whether this strain is perhaps unusual in its occurrence, or is sufficiently widespread to be considered of importance in the epidemiology of this disease, neutralization tests with human gamma globulin were carried out using this strain as well as representatives of the other two antigenic types, Lansing and Brunhilde.<sup>1</sup>

**Methods.** The viruses used have been described in detail before,<sup>1</sup> and the pools used are designated as Brunhilde III, Lansing VIII, and Leon I. These virus pools are of high titer, and consist of 10 or more cords of rhesus monkeys killed on the first day of paralysis. Only lumbar and cervical enlargements were used. Aqueous suspensions were prepared with a Waring blender, and aliquots sealed in glass ampules and stored on dry ice. Titrations of these pools are shown in Table I.

The globulin solution used was a sample supplied by the courtesy of the American Na-

tional Red Cross who distribute it for measles prophylaxis. The sample used was prepared by E. R. Squibb and Sons, and information regarding it was kindly supplied by Dr. J. W. Palmer of the Squibb Company. The human plasma pool used in the preparation of the globulin pool from which this sample was derived totalled 3,000 liters and consisted of the surplus plasma returned to the American Red Cross by the armed forces after the war. The plasma is therefore representative of about 20,000 to 50,000 individuals who contributed blood to the Red Cross during the war. These individuals lived predominantly on the East Coast and in the Great Lakes Area. A small fraction of the plasma was also collected in the Far West. The plasma was originally dried and subsequently reconstituted. The primary fractionation was made following method 6 of Cohn, *et al.*;<sup>4</sup> the subfractionation was made according to method 9 of Oncley, *et al.*<sup>5</sup> The preparation of the globulin fraction from plasma does not appear to result in deterioration of antibody.<sup>6</sup> The effect of prolonged storage of the plasma in the dried state might conceivably have had a detrimental effect on antibody levels, although this seems doubtful in view of the

\* Aided by a grant from The National Foundation for Infantile Paralysis, Inc.

<sup>1</sup> Bodian, D., Morgan, I. M., and Howe, H. A., *Am. J. Hyg.*, 1949, 49, 234.

<sup>2</sup> Kessel, J. F., and Pait, C. F., *Proc. Soc. Exp. Biol. and Med.*, 1949, 70, 315.

<sup>3</sup> Kessel, J. F., Moore, F. J., and Pait, C. F., *Am. J. Hyg.*, 1946, 43, 82.

<sup>4</sup> Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., *J. Am. Chem. Soc.*, 1946, 68, 459.

<sup>5</sup> Oncley, J. L., Melin, M., Richert, D. A., Cameron, J. W., and Gross, P. M., Jr., *J. Am. Chem. Soc.*, 1949, 71, 541.

<sup>6</sup> Enders, J. F., *J. Clin. Invest.*, 1944, 23, 510.

TABLE I.  
Titrations of Virus Pools Used.

Virus and pool No.	Dilutions of cord suspensions in water						
	10-1	10-2	10-3	10-4	10-5	10-6	10-7
Brunhilde III		24/24†	24/25	14/14	4/6	2/6	0/6
Lansing VIII	9/9		13/14	4/6	3/6	0/6	
Leon I	15/15	8/8	16/16	8/8	8/8	6/10	3/10

\* PD50 calculated with paralytic rates taken on a per cent basis.

† Number paralyzed over number inoculated (accumulated inoculations).

TABLE II.  
Neutralization of Poliomyelitis Type Viruses by Human Gamma Globulin.

		Globulin dilutions			NMS*
		10-1	10-2	10-3	
A. 6/7/49	Brunhilde	0/2†	1/2	2/2	3/3
	Lansing	0/2	0/2	2/2	3/3
	Leon	0/2	0/2	2/2	2/3
B. 8/24/49	Brunhilde	0/2	2/4	2/2	
	Lansing	0/1	1/3	2/2	
	Leon	0/2	0/4	2/2	

		Globulin dilutions				Neutralization index‡
		10-1	10-2	10-3	PD50‡	
Total A + B	Brunhilde	0/4	3/6	4/4	10-2	10,000
	Lansing	0/3	1/5	4/4	10-2.4	25,000
	Leon	0/4	0/6	4/4	10-2.5	30,000

\* Normal monkey serum.

† Number monkeys paralyzed over number inoculated.

‡ Calculated by the method of Reed and Muench.<sup>8</sup>

§ Defined here as the antilog of the sum of the exponents of the PD50 of the virus used and the PD50 dilution of globulin. The neutralization index is thus based on the titer of the virus in aqueous solution, and the assumption is made that virus dilutions and globulin dilutions are equivalent.

results to be described.

Our sample consisted of 2 ml of a solution containing 16.8% of gamma globulin, as compared to 0.74% contained in normal plasma, so that the gamma globulin is not only purified but also concentrated to about 23 times the level of normal plasma. It should contain most of the antibodies of adult plasma. The original solution used in the tests to be described was diluted with physiological saline to prepare 1 to 10, 1 to 100, and 1 to 1000 dilutions. These were used immediately for the first series of neutralization tests and the remaining solution stored at 4° C until used in the repeated tests.

The diluted globulin solution was mixed in equal proportions with virus suspensions to prepare the material for inoculation. The mixtures contained 100 PD50 of virus in

0.4 ml, in each instance. The mixtures were allowed to stand at room temperature for 2 hours, and then at 4° C for 2 hours, before inoculation. Each monkey was inoculated into the left thalamus with 0.4 ml of the globulin-virus mixture according to the method described in detail elsewhere.<sup>7</sup>

All monkeys were observed for 3 weeks for signs of poliomyelitis. Animals which did not show typical signs, including severe paralysis, were then prepared for histopathological examination of brain and spinal cord. The results recorded were based on the results of these procedures. No instances of non-paralytic poliomyelitis were revealed by the

<sup>7</sup> Bodian, D., Morgan, I. M., and Schwerdt, C. E., *Am. J. Hyg.*, 1950, 51, No. 1.

<sup>8</sup> Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, 27, 493.

histological studies.

**Results.** Results are shown in Table II. They indicate that the neutralizing capacity of gamma globulin in this sample is very high for each of the 3 antigenic types of poliomyelitis virus. The results of the two sets of tests, made with separate virus aliquots, show agreement and indicate that the concentrated gamma globulin can neutralize about 30,000 PD50 of the Lansing and Leon viruses, assuming that virus and globulin dilutions are comparable. Storage of the globulin solution at 4° C for almost 3 months does not appear to have impaired the neutralizing potency.

**Discussion.** The most interesting result of these tests is that pooled adult gamma globulin may contain as much antibody against Leon virus, as against the representatives of the other two types, which are known to be widespread in distribution.<sup>1,2</sup> Since relatively few strains have thus far been differentiated as to type, it is possible that more representatives of the Leon type will be isolated in the future. The question may be raised as to whether this type may have been more or less restricted to the Far West, where Leon was isolated in 1937, especially since our sample of gamma globulin contained some material from that area. In view of the fact that only a small fraction of the plasma pool was obtained from individuals in the Far West, it is doubtful that the Leon antibody was derived only from this source. Since the titer of Leon antibody was equal to that of the Lansing and superior to the Brunhilde virus, there seems every reason to suppose that the Leon virus type is, or was, of importance in the epidemiology of poliomyelitis. It is of further interest that antibody against Brunhilde virus, the representative of the type currently found to be most prevalent,<sup>1</sup> seemed to be present in somewhat less concentration than antibody against the other two types. It is obvious, however, that this preliminary study should be extended to determine in greater detail not only the geographical distribution of type-specific antibodies in human sera, but also the distribution in time and in various age groups. It is also possible to speculate that viruses of the

Brunhilde type, although more prevalent than those of other types, may be inferior as antigens.

Antibody against Lansing poliomyelitis virus has previously been demonstrated in human gamma globulin.<sup>6,9</sup> The fact that antibodies against all 3 known antigenic types of poliomyelitis virus exist in high concentration in gamma globulin adds further emphasis to the report of Bahlke and Perkins,<sup>10</sup> who found that relatively large amounts of this material were ineffective in the treatment of preparalytic poliomyelitis. Although elaborate controls were exercised in their study, and although it is now clear that high polyvalent antibody levels are present in gamma globulin, their conclusion that serum therapy, in any form, is ineffective in poliomyelitis still seems to require some qualification. First, the virus, or viruses, responsible for the cases they attempted to treat was not identified as to type, so that a remote possibility exists that specific antibody was not present in the gamma globulin used. Moreover, unconcentrated hyperimmune monkey serum contains levels of antibody approximately the same as those in human gamma globulin.<sup>11,12</sup> Preparation of gamma globulin from hyperimmune serum could therefore be expected to produce antibody levels about 20 times greater than those found in human gamma globulin.

**Summary.** A sample of gamma globulin, refined and concentrated about 23-fold from pooled adult human plasma, was tested for neutralizing antibodies against representatives of three distinct antigenic types of poliomyelitis virus, Brunhilde, Lansing, and Leon. The neutralizing potency was high against all three viruses. A dilution of the globulin solution of 1/100 neutralized 100 PD50 of virus in almost every instance. A dilution of 1/1000 failed to neutralize 100 PD50 of virus.

<sup>9</sup> Kramer, S. D., quoted in <sup>10</sup>.

<sup>10</sup> Bahlke, A. M., and Perkins, J. E., *J.A.M.A.*, 1945, 129, 1146.

<sup>11</sup> Morgan, I. M., *J. Immunol.*, 1949, 62, 301.

<sup>12</sup> Howe, H. A., *Am. J. Hyg.*, in press.

# Pteroylglutamic Acid, Ascorbic Acid, and Injectable Liver Extract on Dietary Glycine Toxicity in the Rat.\* (17400)

JAMES S. DINNING, CECILIA K. KEITH, PAUL L. DAY, AND JOHN R. TOTTER.

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Previous work from this laboratory has demonstrated that the growth retardation resulting from inclusion of sodium benzoate in a purified diet for rats may be in part overcome by the addition of pteroylglutamic acid (PGA) in the diet.<sup>1</sup> Experiments were next designed to study the effect of feeding high glycine diets to rats. In confirmation of the work of Hier *et al.*<sup>2</sup> it was found that rats receiving a purified diet containing 10% glycine grew at a subnormal rate; hence the effects of supplementing this diet with PGA, injectable liver extract, and ascorbic acid were studied. While these experiments were in progress Martel and Gingras<sup>3</sup> reported that the feeding of a 10% glycine diet to rats resulted in growth suppression, mild leucopenia, and an elevated creatinuria, all of which responded to PGA.

**Experimental.** Six groups of weanling Sprague-Dawley rats, 4 males and 4 females in each group, were fed the diets given in Table I. The rats were fed *ad libitum* and food intake was measured. Complete blood counts were made on the 37th and 46th days of the experiment. The differences in weight gains among the various groups were statistically treated by the analysis of variance method.<sup>4</sup>

**Results and Discussion.** The growth curves are given in Fig. 1. It may be ob-

served that the inclusion of 10% glycine in the purified diet resulted in a greatly decreased rate of growth. PGA markedly improved the growth of rats receiving the high glycine diet. The addition of liver extract to the high glycine diet resulted in some growth improvement but less than that produced by PGA. The liver extract used was assayed for PGA and found to contain 2.62  $\gamma$  per cc; this amounted to approximately 0.2  $\gamma$  of PGA per rat per day. This and the fact that the group receiving PGA plus liver extract grew no faster than those receiving PGA alone would indicate that some of the growth improvement resulting from the liver extract may have been due to its content of PGA.

Johnson and Dana<sup>5</sup> have presented data to indicate that ascorbic acid increased the rate of growth and induced a blood response in rats fed sulfasuxadine. Woodruff and Darby<sup>6</sup> have reported that the inclusion of PGA in the diet of scorbutic guinea pigs reduced the excretion of tyrosine metabolites. Such data indicate a similarity in function of PGA and ascorbic acid and for this reason one group of rats was fed the high glycine diet plus ascorbic acid. It may be seen from the growth curves that ascorbic acid had little effect on the growth of the rats. The difference in rate of gain between the group receiving ascorbic acid and the group receiving high glycine alone was not statistically significant.

Weight gains per gram of food eaten were calculated for the various groups and the average values were: control 0.295, high glycine 0.152, high glycine plus PGA 0.282, high glycine plus liver extract 0.205, high

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1 Totter, John R., Amos, Esther S., and Keith, Cecilia K., *J. Biol. Chem.*, 1949, **178**, 847.

2 Hier, Stanley W., Graham, Claire E., and Klein, David, *Proc. Soc. Exp. Biol. and Med.*, 1944, **50**, 187.

3 Martel, F., Page, E., and Gingras, R., *Rev. Canadienne de Biologie*, 1947, **VI**, 802.

4 Snedecor, George W., *Statistical methods applied to experiments in agriculture and biology*, The Iowa State College Press, 4th ed., 1946.

5 Johnson, B. Connor, and Dana, Ann S., *Science*, 1948, **108**, 210.

6 Woodruff, Calvin W., and Darby, William J., *J. Biol. Chem.*, 1948, **172**, 851.

TABLE I.  
Composition of Experimental Diets.

Dietary component	Groups					
	I	II	III	IV	V	VI
Casein, %	18	18	18	18	18	18
Sucrose, %	75	65	65	65	65	65
Crisco, %	3	3	3	3	3	3
Cod liver oil, %	2	2	2	2	2	2
Salt mix*, %	2	2	2	2	2	2
Glycine, %	0	10	10	10	10	10
PGA—mg/100 g	0	0	0.5	0	0.5	0
Liver extr.—cc/kilo	0	0	0	8	8	0
Ascorbic acid—mg/100 g	0	0	0	0	0	100

The liver extract was an anti-pernicious anemia concentrate containing 15 units per cc. The following vitamins were added per 100 g to all diets:

	mg		mg
Thiamine	0.5	Choline chloride	10.0
Riboflavin	0.5	Pyridoxine hydrochloride	0.5
Nicotinic acid	2.0	Biotin	0.005
i-Inositol	10.0	Vit. K	0.025
Calcium pantothenate	1.0		

\* Hubbell, R. B., Mendel, L. B., and Wakeman, A. J. *J. Nutr.*, 1937, 14, 273.

glycine plus PGA plus liver extract 0.291, and high glycine plus ascorbic acid 0.213. With the exception of reversal between the ascorbic acid and liver extract groups, efficiency of gain ranked the same in the various groups as did total weight changes.

The hematological data are presented in Table II. Data for males and females are

given separately only in Groups IV and V, since there were no sex differences in the other groups. The feeding of the high glycine diet did not produce a significant leucopenia in these rats when compared to the controls; however, addition of PGA to this diet did elevate the white cell count. Addition of liver extract and liver extract plus PGA to the high glycine diet resulted in an increased white count in male rats but was without effect in the females.

Although the red cell counts of rats receiving the high glycine diet were lower than the control group, none of the supplements tested were effective in restoring the counts to the level of the controls. Hemoglobin values were also reduced by the high glycine diet and were improved only by the addition of the combination of PGA plus liver extract or by ascorbic acid. There were no consistent differences in hematocrit or differential white cell count among the various groups. The rats receiving ascorbic acid were found to have a higher mean corpuscular volume than any of the other groups. It may also be noted that rats receiving ascorbic acid had the lowest red count of any of the groups, but had a higher hemoglobin level than any of the others except the control group and the

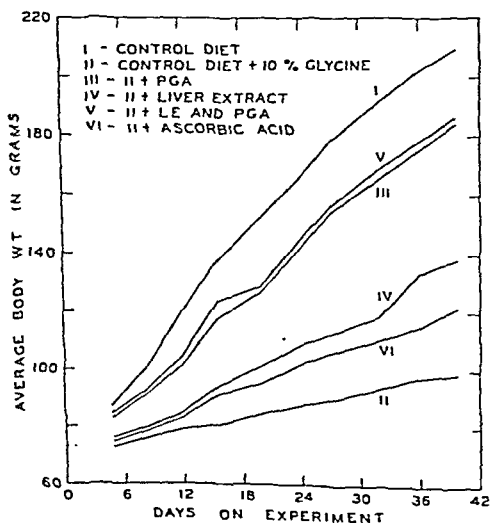


Fig. 1.

Growth curves for rats receiving the various diets.

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**Results and Discussion.** The growth curves are given in Fig. 1. It may be ob-

served that the inclusion of 10% glycine in the purified diet resulted in a greatly decreased rate of growth. PGA markedly improved the growth of rats receiving the high glycine diet. The addition of liver extract to the high glycine diet resulted in some growth improvement but less than that produced by PGA. The liver extract used was assayed for PGA and found to contain 2.62  $\gamma$  per cc; this amounted to approximately 0.2  $\gamma$  of PGA per rat per day. This and the fact that the group receiving PGA plus liver extract grew no faster than those receiving PGA alone would indicate that some of the growth improvement resulting from the liver extract may have been due to its content of PGA.

Johnson and Dana<sup>5</sup> have presented data to indicate that ascorbic acid increased the rate of growth and induced a blood response in rats fed sulfasuxadine. Woodruff and Darby<sup>6</sup> have reported that the inclusion of PGA in the diet of scorbutic guinea pigs reduced the excretion of tyrosine metabolites. Such data indicate a similarity in function of PGA and ascorbic acid and for this reason one group of rats was fed the high glycine diet plus ascorbic acid. It may be seen from the growth curves that ascorbic acid had little effect on the growth of the rats. The difference in rate of gain between the group receiving ascorbic acid and the group receiving high glycine alone was not statistically significant.

Weight gains per gram of food eaten were calculated for the various groups and the average values were: control 0.295, high glycine 0.152, high glycine plus PGA 0.282, high glycine plus liver extract 0.205, high

\* Research paper No. 889, Journal Series, University of Arkansas. This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

<sup>1</sup> Totter, John R., Amos, Esther S., and Keith, Cecilia K., *J. Biol. Chem.*, 1949, **178**, 847.

<sup>2</sup> Hier, Stanley W., Graham, Claire E., and Klein, David, *Proc. Soc. Exp. Biol. and Med.*, 1944, **56**, 187.

<sup>3</sup> Martel, F., Page, E., and Gingras, R., *Rev. Canadienne de Biologie*, 1947, **VI**, 802.

<sup>4</sup> Snedecor, George W., *Statistical methods applied to experiments in agriculture and biology*, The Iowa State College Press, 4th ed., 1946.

<sup>5</sup> Johnson, B. Connor, and Dana, Ann S., *Science*, 1948, **108**, 210.

<sup>6</sup> Woodruff, Calvin W., and Darby, William J., *J. Biol. Chem.*, 1948, **172**, 851.

# The Effect of Staphylococcal Enterotoxin upon the Frog, (*Rana pipiens*). (17401)

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Significant advances in the study of staphylococcal food poisoning have been extremely difficult because of the lack of a reliable diagnostic test. Bioassay methods have been described using human volunteers, monkeys, suckling pigs, and kittens and cats,<sup>1-4</sup> none of which has proved to be entirely satisfactory. The ability of an enterotoxin produced from a strain of *Micrococcus pyogenes* var. *aureus* to elicit a vomit reaction in a test animal has been considered evidence of a positive test. Animals such as ruminants, horses, and rodents lack the appropriate coordinating vomit mechanism<sup>5</sup> and hence the usual laboratory animal (rabbit, guinea pig, rat, and mouse) has been of little value. The common frog, *Rana pipiens*, was chosen for this study because it has the ability to exhibit reverse peristalsis of the stomach,<sup>6</sup> is readily available, is inexpensive, and is easily maintained under laboratory conditions.

**Procedures and results.** Preliminary experiments were carried out, using a filtrate prepared from a known enterotoxigenic strain of *Micrococcus pyogenes* var. *aureus*.<sup>\*</sup> Dolman's<sup>7</sup> technique was followed in detail in the preparation of this and all subsequent filtrates. Only healthy, well-nourished frogs were used.

<sup>1</sup> Jordan, E. O., *J.A.M.A.*, 1931, **97**, 1704.

<sup>2</sup> Jordan, E. O., *Proc. Soc. Exp. Biol. and Med.*, 1931, **29**, 161.

<sup>3</sup> Hopkins, E. W., and Poland, E. F., *Food Res.*, 1942, **7**, 414.

<sup>4</sup> Dolman, C. E., *Canad. J. Pub. Health*, 1926, **27**, 489.

<sup>5</sup> Meyer, H. H., and Gottlieb, R., *Experimental Pharmacology*, J. B. Lippincott, Philadelphia, Pa., 1926, 2nd ed.

<sup>6</sup> Alvarez, W. C., *An Introduction to Gastroenterology*, P. B. Hoeber, N. Y., 1940, 3rd ed.

<sup>7</sup> Dolman, C. E., *Canad. J. Pub. Health*, 1940, **51**, 68.

\* Strain No. 8 obtained through the kindness of Dr. Gail Dack, Department of Bacteriology and Parasitology, University of Chicago.

Filtrates were first administered either parenterally (into the dorsal lymph sac) or orally in No. 00 gelatin capsules. Satisfactory results were obtained, however, by the simpler procedure of direct feeding with a medicine dropper. This was accomplished by opening the frog's mouth with large, blunt-ended forceps, and then, while holding the mouth open by a thumb inserted near the angle of the jaw,<sup>8</sup> slowly inserting the dropper through the esophageal sphincter. Thus, the filtrate was placed directly into the frog's stomach. The frog under test was placed in an aquarium jar containing a small amount of water and was observed over a period of hours. A positive test for enterotoxin is shown by the production of a series of reactions termed for the purpose of this discussion a "spasm", for although reverse peristalsis of the stomach could be demonstrated, vomiting of the stomach contents never occurred.

A spasm is initiated by a series of swallowing motions (retraction of the eyeballs), a slowly repeated gaping of the mouth, a gradual change in the tone of the skin color, a distension of the abdominal wall, a distinctive sitting position (the forelegs at full height and the back raised so that it parallels the floor of the aquarium). This takes place within 3 to 10 minutes and reaches a climax when the spasm is completed by a slow-motion rubbing of the abdominal wall with a hind-foot followed by a wiping of the face and mouth with a fore limb. This occurs on both sides of the body and may last from 3 to 15 minutes. Gradually, mucous is expelled from the mouth, abdominal distension is decreased and the frog assumes a natural sitting position. Gaping of the mouth may continue for some time after the other characteristic body motions have stopped. The rapidity with which the spasm follows the

<sup>8</sup> Rose, S. Meryl, personal communication, 1949, Department of Zoology, Smith College.



TABLE II.  
Average Hematological Data for Rats Receiving the Various Diets.

Group	Wbc., 10 <sup>3</sup> /mm <sup>3</sup>	Reticulocytes, % of rbc.	Granulocytes, % of wbc.	Lymphocytes, % of wbc.	Rbc., 10 <sup>6</sup> /mm <sup>3</sup>	Hb., g/100 cc	Vol. packed Rbc. cc/100 cc	Mean corpuscular vol., microns <sup>3</sup>
I. Control	17.9	2.6	11	89	7.29	14.9	51	70
II. High glycine	16.4	2.2	17	83	6.75	13.9	49	74
III. High glycine + PGA	19.1	2.2	10	90	6.70	13.2	49	74
IV. High glycine + L.E.	20.7 14.5	2.3 2.5	20 12	80 88	6.29 6.09	13.1 13.9	46 49	73 81
V. High glycine + PGA + L.E.	26.5 17.5	3.5 3.6	17 9	83 91	6.80 6.27	14.9 14.6	51 50	75 79
VI. High glycine + ascorbic acid	16.4	3.1	13	87	6.20	14.1	50	82

group receiving liver extract plus PGA.

There is not sufficient evidence available at present to permit an explanation of the growth-promoting effect of PGA when added to a high glycine diet for rats. Holland and Meinke<sup>7</sup> have presented evidence which indicates that PGA may function in the synthesis of serine by *Streptococcus faecalis*. It has been shown by Sakami<sup>8</sup> that glycine is converted to serine and then to glycogen by the rat. It is possible that this conversion may be a factor in explanation of our results. Experiments are in progress to study the effects of PGA on the conversion of glycine to serine by liver homogenates.

**Summary.** Weanling albino rats were fed a standard purified diet, and a similar diet containing 10% glycine replacing an equal amount of sucrose. Groups of rats were given the glycine-containing diet supplemented respectively with PGA, injectable liver extract, PGA plus liver extract, and ascorbic acid. The growth of rats receiving the high glycine diet was greatly reduced as compared to the control group. Supplementation of the high glycine diet with PGA and with PGA plus liver extract resulted in a marked improvement in growth rate; liver extract alone was less effective; and ascorbic acid did not significantly improve rate of growth of rats receiving the 10% glycine diet. Rats receiving the high glycine diet did not develop a marked leucopenia when compared with control rats; however, supplementation of this diet with PGA resulted in an increased white count while supplementation with liver extract and with liver extract plus PGA resulted in an increased white count in male rats but was without effect in females. The mean corpuscular volume was found to be highest in rats receiving the high glycine diet plus ascorbic acid.

<sup>7</sup> Holland, Bryant R., and Meinke, W. W., *J. Biol. Chem.*, 1949, **178**, 7.

<sup>8</sup> Sakami, W., *J. Biol. Chem.*, 1948, **170**, 995.

as applicable to animate as to inanimate objects. Nevertheless much is unknown concerning the chain of events which leads to the eventual dissolution or loss of the integrity of a system.

Specific agents are known which produce in a mammal a syndrome comparable to that seen following radiation. For example, it is possible with folic acid antagonists to produce epilation, lymphopenia, anemia, hemorrhage, diarrhea, anorexia, malaise, wasting and eventual death in much the same sequence as occurs with radiation. This can be done with concentrations of these substances so low in body fluids that they avoid chemical detection.

It is possible that the indiscriminate production of ion pairs and excitation of atoms in animals by means of radiation may be followed by the recombination of ions in such a manner that the probability of the formation of a specific substance causing the sequence of events seen in radiation illness is present. Evidence for toxic recombinations has been gained from the study of the radiation of water *in vitro*.<sup>1,2</sup> Other simple *in vitro* experiments have led to the idea of direct and indirect action.<sup>3</sup> A direct action is one in which ionization produced within the molecule causes the effect while an indirect action is one in which the effect occurs because of ionization produced in surrounding material. Apart from this consideration of whether a toxic substance is produced directly or indirectly, a second, perhaps less fundamental question has concerned investigators: *Is the toxic material during and after formation free to move from one part of the animal's system to another and act there?*

The conviction that the production of a specific harmful substance could not occur without being conveyed throughout the animal's system has led to cross circulation experiments in which the vascular flow of a non-treated animal was connected to that of a radiated animal.<sup>4-8</sup> In general these experiments have been performed with complete

disregard for the magnitude of the vascular exchange or with some regard but little evaluation. It is particularly important to realize that negative results of cross circulation radiation experiments are worthless so far as disproving an hypothesis that radiation damage is caused by a circulating agent. Moreover similar physical considerations apply to transfer within a single animal. A physico-mathematical consideration of cross circulation experiments has been presented in which it is shown that the distribution of a substance within a system is a function of at least two turnover rates, (1) that of the blood flow to the area and (2) that of the metabolite.<sup>9</sup>

Early investigators had much difficulty with the parabiotic technique—indeed some of them admitted to more operative failures than successes and with a successful operation there was always the question of the efficacy of the inosculation. Zacherl irradiated parabiotic rats with a lethal and unspecified amount of X-ray.<sup>4</sup> He made no statement concerning the specific nature of the shielding afforded the protected members of the pair. He observed concomitant fall in the temperature and white blood count of the members of the pair; however, the fall of both the white blood count and the temperature was not as great in the protected member. He gave little consideration to the effect of dilution which would occur with mixing. He was convinced of a radiation toxin. Behnes' experiments on parabiotic guinea pigs are less clearly described.<sup>5</sup> Woenckhaus also was convinced that a radiation toxin was produced, but his

<sup>4</sup> Zacherl, H., *Beitrag zur Allgemeinwirkung der Röntgenstrahlen Strahlentherapie*, Band, 1926, **23**, 272.

<sup>5</sup> Behne, *Deutsche Med. Wochens.*, 1920, **46**, 223.

<sup>6</sup> Woenckhaus, E., *Arch. f. Exp. Path. und Pharmak.*, 1930, **150-152**, 183.

<sup>7</sup> Lawrence, J. S., Dowdy, A. H., and Valentine, W. N., Atomic Energy Commission Declassified Document No. MDDC-853, 1947.

<sup>8</sup> Barnes, W. A., and Furth, O. B., *Am. J. Roent.*, 1943, **49**, 662.

<sup>9</sup> Huff, R. L., Trautman, R., and Van Dyke, D. C., to be published.

<sup>1</sup> Risse, O., *Z. Phys. Chem. A*, 1929, **140**, 133.

<sup>2</sup> Fricke, H., *J. Chem. Phys.*, 1934, **2**, 556.

<sup>3</sup> Lea, D. E., *Action of Radiations on Living Cells*, chap. 2, Macmillan Co., 1947.

feeding of the filtrate is apparently dependent upon individual susceptibility and enterotoxin potency. Reactions have been observed within a range of 45 minutes to 7 hours. After the spasm has subsided, further reactions may be elicited, for at least a 10-day period, by feeding the frog. Therefore, it is possible to feed and then leave the animal until the next morning when a meal is given. A typical spasm occurs within 3 to 4 hours.

Such typical reactions have been obtained with other known enterotoxigenic strains of *Micrococcus pyogenes* var. *aureus*,<sup>†</sup> while no such reaction has ever been observed in frogs fed with filtrates prepared from known negative strains of *Micrococcus pyogenes* var. *aureus* No. 902 and 64, *Escherichia coli*, *Porteus vulgaris*, and sterile culture medium. The results of these tests are summarized in Table I.

A series of frogs was fed known enterotoxigenic filtrates in an effort to show reverse peristalsis of the stomach. At the height of a spasm the animal was decapitated. In each frog studied, the esophageal sphincter was relaxed and the stomach was observed in reverse motion. Controls included unfed frogs, those fed hamburg, and those fed sterile

<sup>†</sup> Enterotoxigenic strains Nos. 432 and 422 and non-enterotoxigenic strains Nos. 902 and 64 were obtained through the kindness of Dr. Glenn Slocum, Food and Drug Administration, Washington, D.C.

TABLE I.  
Effect of Enterotoxigenic and Non-Enterotoxigenic Filtrates Upon the Frog (*Rana pipiens*).

Organism used in filtrate preparation	Total No. of frogs tested	Reaction observed
<i>Micrococcus pyogenes</i> var. <i>aureus</i>		
No. 161	28	Spasms
No. 8	8	"
No. 432	21	"
No. 422	5	"
No. 64	18	None
No. 902	5	"
<i>Micrococcus pyogenes</i> var. <i>albus</i>	9	"
<i>Escherichia coli</i>	6	"
<i>Proteus vulgaris</i>	3	"
Sterile culture medium	29	"

culture medium. One each of these was sacrificed with each test frog. In no control was any reverse motion of the stomach observed.

**Summary.** Staphylococcal enterotoxins which gave positive results with the kitten test were capable of producing spasms when fed to frogs. It would appear from the results summarized in Table I, that the frog test was specific for no false positive reactions were noted. It is not possible to use a frog more than once when a positive reaction has been obtained and further study is in progress to determine the nature of the injury to the digestive mechanism.

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### Epilation in the Non-Irradiated Member of Parabiotically United Rats.\* (17402)

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Despite the lack of our ability to explain this phenomenon—the loss of hair in the non-irradiated members of parabiotically united

rats—it was thought worthy of record as an observation.

The relatively few known fundamental reactions involved when either particulate or electromagnetic radiation reacts with matter are described in the standard textbooks of Atomic Physics. These explanations are fully

\* This work was supported in part by the Atomic Energy Commission.

<sup>†</sup> Atomic Energy Commission Postdoctorate Research Fellow.

expectancy. In none of them is observed the wasting and anemia so often described for parabiotic rats. The vascular exchange rate is .6% of the blood volume per minute.<sup>10</sup> Litter mates were surgically united (coelio-anastomosis) at 20-30 days of age.

After the pairs of Groups I and II had been joined for 2 months the members of Groups I and III were radiated alternately on the same day. Precisely: one pair was placed in the chamber, one animal receiving 27 roentgens and the other 900 roentgens; then 2 single animals were placed in the container one receiving 27 roentgens and the other 900 roentgens, etc. Following radiation 2 animals from each of the 3 groups were maintained in the same cage. Treatment with DDT was carried out weekly.

**Results.** For the most part the protected members of the radiated pairs and the single

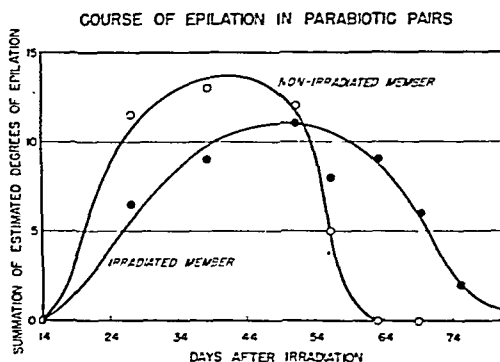


FIG. 3.

animals receiving only 27 roentgens showed no gross signs of radiation injury. However, about 4 weeks following radiation both members of the six pairs in Group I started to lose their hair. Such a pair is shown in Fig. 2. The epilation was observed simultaneously but was first much more marked in the protected members. Rough estimation of the degree of epilation (1 to 4+) was made and the tabulation of these estimations for the 6 pairs, Fig. 3, gives the course of the epilation in the 2 animals. The protected animals were affected to a greater degree but for a shorter time than the unprotected members. Neither the single controls, receiving 27 roentgens, nor the non-radiated parabionts of Group II showed any sign of epilation. The single controls receiving 900 roentgens died within one week after radiation. The protection afforded the radiated parabiont will be discussed in a later communication.

**Discussion and Conclusion.** Parabiotically united rats influence their partners by the mixing of their body fluids through their common circulation. Therefore, an effect such as epilation must result from the transfer of material from one member to the other. Whether the epilation represents the passage of physiologically active material from the radiated to the non-radiated or is a manifestation of loss of material from the non-irradiated to the irradiated is not demonstrated.



FIG. 2.

Parabiotic rats showing epilation of both (A) irradiated and (B) non-irradiated members.

<sup>10</sup> Van Dyke, D. C., Huff, R. L., Evans, H. M., *Stanford Med. Bull.*, 1948, 6, 271.

experiments are subject to the same criticism as Zacherl's.<sup>6</sup> Lawrence *et al.*<sup>7</sup> reviewed the literature concerning the evidence for a circulating radiation toxin and concluded that the question was not settled. They set out to confirm or deny the presence of such a substance by cross-circulating cats (carotid to carotid anastomoses) at various time periods following radiation of one with 1500 r X-ray for a duration of approximately 10 hours. In spite of the fall in absolute lymphocyte count which occurred during and after the cross circulation in the non-radiated member they concluded that their results did not support the theory of a radiation toxin. Exact interpretation of their experiments requires that the turnover rates of white cells and of the blood flow between the animals be known. Furth and Barnes<sup>8</sup> performed an extensive experiment on parabiotic mice which included much microscopic tissue examination. The non-radiated members of the pairs showed non-specific changes which were similar to but much less than in the radiated animal. They mentioned that the parabiotic combination offered protection from radiation.

In this laboratory an experiment was planned to determine the magnitude of the protection from radiation afforded rats in parabiosis. During the course of this study it was noted that both the radiated and non-radiated members of a pair showed epilation; starting about 4 weeks after radiation and lasting 6 weeks.

**Methods.** The apparatus used for radiation is shown in Fig. 1. It consisted of a lead chamber in juxtaposition with a lucite chamber between which was a lead divider except in the area corresponding to the connecting tissue of the pairs. The dosage given was determined by inserting a lucite phantom, which has approximately the x-ray absorption equivalent of tissue. Victoreen ionization chambers were placed in holes drilled in the center of the phantom, and a third was placed in an attached lead box to serve as a monitor. Radiation conditions were 200 K.V., 15 ma, 0.5 mm Cu and 1.0 mm Al filters, 37.5 cm tube distance, rate 40 r/min. The time necessary to deliver the desired dose was determined with the ionization chambers in the plastic

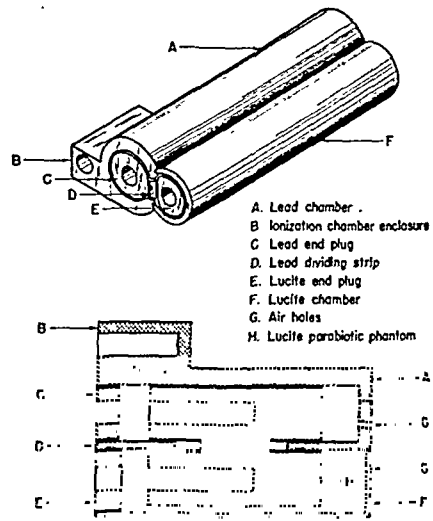


FIG. 1.

Chamber for exposing parabiotic rats to radiation.

phantom. The reading in the lead box was found to be a constant fraction of the dose received inside the phantom, and this reading was used later to check the dose given the animals. The dose delivered inside the phantom protected by the lead chamber was found to be 3% of that given the unprotected member. Readings were made inside the lead chamber by means of the Victoreen Ionization Chambers and also with x-ray film to insure the absence of large amounts of soft radiation from scatter.

Three groups of animals were prepared for the experiment. Group I consisted of 6 parabiotic pairs which were subjected to radiation, Group II was 6 parabiotic pairs not radiated and serving as controls to parabiosis and Group III was composed of 12 single animals radiated in the same manner as those of Group I. All animals were of approximately the same weight so as to insure comparable depth dose. The rats were females of the Slonaker strain which had been propagated by brother and sister inbreeding for 50 generations. This degree of inbreeding should result in an almost homozygous genetic pattern which would tend to eliminate the antigen specificity of their nuclear proteins. Pairs prepared from these animals remain in perfect health for the normal life

TABLE I.  
 Ratings of Nests on Each Day in the Attic Room.  
 (Rating of shocked rats 1-7 and controls 1-4 was discontinued on the 10th day, post shock).

Day in attic	Shock	Daily ratings on a scale of 0-4															
		Shocked animals (Nos.1-12)												Control animals (Nos. 1-9)			
		1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4
1	9th	0	0	0	0	0	0	0	0	0	0	0	0	1	4	4	3
2	10th	0	0	0	2	1	0	0	0	0	0	0	0	4	4	4	3
3	0	1	0	1	3	1	0	1	0	0	0	0	1	3	4	4	4
4	0	1	0	0	3	0	1	2	1	0	1	1	1	4	4	4	4
5	0	1	1	0	3	0	1	3	0	1	1	2	1	4	4	4	4
6	0	2	1	2	4	1	1	3	1	0	2	3	2	4	4	4	4
7	0	2	2	3	4	1	2	4	1	0	2	3	2	4	4	4	4
8	0	4	3	3	4	2	2	4	2	1	3	4	2	4	4	4	4
9	0	4	4	4	4	3	3	4	2	1	3	3	2	4	4	4	4
10	0	4	4	4	4	3	3	4	2	2	3	4	3	4	4	4	4
11	0	-	-	-	-	-	-	-	1	0	1	2	2	-	-	-	-
12	0	-	-	-	-	-	-	-	1	3	2	2	4	-	-	-	-
13	0	-	-	-	-	-	-	-	3	3	3	4	4	-	-	-	-
14	0	-	-	-	-	-	-	-	2	2	3	3	4	-	-	-	-
15	0	-	-	-	-	-	-	-	2	3	2	3	4	-	-	-	-
16	0	-	-	-	-	-	-	-	1	2	1	3	4	-	-	-	-
17	0	-	-	-	-	-	-	-	4	3	2	3	4	-	-	-	-
18	0	-	-	-	-	-	-	-	3	3	2	3	4	-	-	-	-
19	0	-	-	-	-	-	-	-	1	3	2	3	4	-	-	-	-

score.

No further shocks were administered. As can be seen in Fig. 1, shocked animals gradually began to build better nests, but it was not until the fifth day after the last shock (seventh day in the attic room) that the nests of the convulsed animals began to approach those of the control rats in structure and composition. By the seventh day, post shock, the nests of 4 of the experimental animals could not be distinguished from those of the control group, but those of the other 8 were slightly inferior. By the tenth day there were only minor differences on the part of a few of the shocked rats.

On the tenth day, post shock, the nests of 5 animals from each group were removed and fresh nesting materials provided. Differences between shocked and non-shocked animals showed up again. By the following day (the eleventh day) the control animals had rebuilt good nests while the convulsed animals had built nests which deserved ratings no better than 2. Improvement was rapid, however, so that by 48 hours later, nests which were rated 3 and 4 were built. This rating surpassed their previous highest rating and nearly equalled that of control group. There-

after no constant differences were found.

Rating of 5 of the shocked rats and five of the controls was continued until the nineteenth day, post shock, when the same group of psychology students were told that the positions of the animals had been changed. Using the same criteria for rating, they were unable to distinguish between the two groups.

*Discussion.* The study reported in this paper combines the findings of 2 experimenters using the same procedures. The behavior of the shocked animals in both instances was so identical as to warrant reporting them as one study. The fact that the same results were obtained by 2 experimenters working one year apart lends support to the validity of the findings. We may conclude that electroconvulsive shocks disturb nest building activity in the male albino rat in an environment in which the diurnal range of temperatures is from 52°-75°F, and usually below 65°F. Furthermore, the disruptive effects of convulsive shocks on nest building activity of the male rat demonstrates that the effects in the female described by Rosvold<sup>1</sup> may be common to both sexes, and that they are not necessarily the result of the combined factors of shock and maternity.

# Effect of Electroconvulsive Shocks on Nest Building in the Male Albino Rat.\* (17403)

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*From the Department of Psychology, McGill University, Montreal, and the Department of Psychology, Stanford University, California.*

In an investigation of the effect of electroconvulsive shock on maternal behavior, Rosvold<sup>1</sup> studied the change in nest building activity in the female albino rat. His data show definite interference with nest building activity, varying in degree with the length of time after parturition that the series of convulsive shocks is introduced.

Since Kinder<sup>2</sup> has shown that males build nests when environmental temperatures are low, it is possible to test the effects of electroconvulsive shocks on nesting activity in animals free from the influence of the physiological conditions due to pregnancy and maternity. Should the shock disrupt the nest building of males, it could be argued first, that shock influences some factor common to both sexes rather than one specific to the female, and second, that the effect in the females is not the result of the combined stress of the convulsive shock and maternity.

**Procedure.** Twenty-one adult male albino rats approximately 100 days old were selected from the Stanford laboratory stock. Twelve of these were shocked each day for 9 days, after which they were moved into a relatively cold attic room (diurnal range, 52°-75°F; usually below 65°F) for observation of their nest building activity. Nine, not shocked but otherwise treated like the experimental animals, served as controls. Each male was placed in a cage similar to those used for the females of Rosvold's<sup>1</sup> study. Shavings and paper were provided for nesting. A tenth shock was given about 24 hours after the rats had been in the attic. Thereafter they

were undisturbed except for routine care and observation, and the nests were left intact.

A five point scale was used to rate their nests. The points were defined as 0 for no use of nesting materials, 1 if the paper had been moved to a preferred sleeping place, 2 if all the paper had been gathered, 3 if a bowl had been formed with paper, and 4 if the best possible nest had been built of shavings and all of the paper.

The electroconvulsive shocks were induced by administering 45 milliamperes for 0.2 second through alligator clips attached to the rat's ears. This strength of current invariably induced a complete tonic-clonic convulsion.

**Results.** During the *first* day in the cold room the temperature did not go above 64°F which, according to Kinder,<sup>2</sup> should have afforded ample thermal incentive for nest building. Table I gives the ratings assigned to the nests on each observation day in the attic room. The control animals began nesting immediately, and after 6 hours 2 had built nests that were rated as 1, one rated as 2, two as 3, four as 4. Not one of the shocked animals (having received 9 shocks) built a nest on the first day in the attic room.

On the *second* day the shocked males experienced the tenth convulsion while the controls were removed from the cages for a similar length of time. On this day the control group improved their nests with the result that 6 were rated 4, two rated 3, and one, 2. Most of the shocked animals did very little nest building. One gathered some paper into a preferred corner and was rated 1, and another built a nest base which was rated 2. Twenty-five university students taking a course in comparative psychology distinguished between the experimental and control groups very definitely, giving the shocked group less than 15% of a perfect score and the control group more than 80% of a perfect

\* Financed from a Scottish Rite Research Fund administered at Stanford University by Calvin P. Stone.

† Now at Yale University, Departments of Psychology and Psychiatry.

<sup>1</sup> Rosvold, H. E., *J. Comp. Physiol. Psychol.*, 1949, **42**, 118.

<sup>2</sup> Kinder, E. F., *J. Exp. Zool.*, 1927, **47**, 117.

TABLE I.

Ratings of Nests on Each Day in the Attic Room.

(Rating of shocked rats 1-7 and controls 1-4 was discontinued on the 10th day, post shock).

		Daily ratings on a scale of 0-4																				
Day in attic	Shock	Shocked animals (Nos.1-12)												Control animals (Nos. 1-9)								
		1	2	3	4	5	6	7	8	9	10	11	12	1	2	3	4	5	6	7	8	9
1	9th	0	0	0	0	0	0	0	0	0	0	0	0	1	4	4	3	1	3	2	4	4
2	10th	0	0	0	2	1	0	0	0	0	0	0	0	4	4	4	3	2	4	3	4	4
3	0	1	0	1	3	1	0	1	0	0	0	0	1	3	4	4	4	2	3	4	4	4
4	0	1	0	0	3	0	1	2	1	0	1	1	1	4	4	4	4	3	3	4	4	4
5	0	1	1	0	3	0	1	3	0	1	1	2	1	4	4	4	4	3	3	4	4	4
6	0	2	1	2	4	1	1	3	1	0	2	3	2	4	4	4	4	3	3	4	3	4
7	0	2	2	3	4	1	2	4	1	0	2	3	2	4	4	4	4	3	3	4	3	4
8	0	4	3	3	4	2	2	4	2	1	3	4	2	4	4	4	4	3	3	4	3	4
9	0	4	4	4	4	3	3	4	2	1	3	3	2	4	4	4	4	3	4	4	4	3
10	0	4	4	4	4	3	3	4	2	2	3	4	3	4	4	4	4	3	3	4	4	4
11	0	-	-	-	-	-	-	-	1	0	1	2	2	-	-	-	-	3	3	3	3	4
12	0	-	-	-	-	-	-	-	1	3	2	2	4	-	-	-	-	3	3	4	3	3
13	0	-	-	-	-	-	-	-	3	3	3	4	4	-	-	-	-	4	2	4	3	4
14	0	-	-	-	-	-	-	-	2	2	3	3	4	-	-	-	-	3	2	3	3	3
15	0	-	-	-	-	-	-	-	2	3	2	3	4	-	-	-	-	4	3	3	3	3
16	0	-	-	-	-	-	-	-	1	2	1	3	4	-	-	-	-	3	3	2	3	3
17	0	-	-	-	-	-	-	-	4	3	2	3	4	-	-	-	-	4	3	4	3	4
18	0	-	-	-	-	-	-	-	3	3	2	3	4	-	-	-	-	3	2	4	3	3
19	0	-	-	-	-	-	-	-	1	3	2	3	4	-	-	-	-	4	3	4	3	4

score.

No further shocks were administered. As can be seen in Fig. 1, shocked animals gradually began to build better nests, but it was not until the fifth day after the last shock (seventh day in the attic room) that the nests of the convulsed animals began to approach those of the control rats in structure and composition. By the seventh day, post shock, the nests of 4 of the experimental animals could not be distinguished from those of the control group, but those of the other 8 were slightly inferior. By the tenth day there were only minor differences on the part of a few of the shocked rats.

On the tenth day, post shock, the nests of 5 animals from each group were removed and fresh nesting materials provided. Differences between shocked and non-shocked animals showed up again. By the following day (the eleventh day) the control animals had rebuilt good nests while the convulsed animals had built nests which deserved ratings no better than 2. Improvement was rapid, however, so that by 48 hours later, nests which were rated 3 and 4 were built. This rating surpassed their previous highest rating and nearly equalled that of control group. There-

after no constant differences were found.

Rating of 5 of the shocked rats and five of the controls was continued until the nineteenth day, post shock, when the same group of psychology students were told that the positions of the animals had been changed. Using the same criteria for rating, they were unable to distinguish between the two groups.

*Discussion.* The study reported in this paper combines the findings of 2 experimenters using the same procedures. The behavior of the shocked animals in both instances was so identical as to warrant reporting them as one study. The fact that the same results were obtained by 2 experimenters working one year apart lends support to the validity of the findings. We may conclude that electroconvulsive shocks disturb nest building activity in the male albino rat in an environment in which the diurnal range of temperatures is from 52°-75°F, and usually below 65°F. Furthermore, the disruptive effects of convulsive shocks on nest building activity of the male rat demonstrates that the effects in the female described by Rosvold<sup>1</sup> may be common to both sexes, and that they are not necessarily the result of the combined factors of shock and maternity.



## HYPOTENSIVE ACTION OF INFLUENZA VIRUS

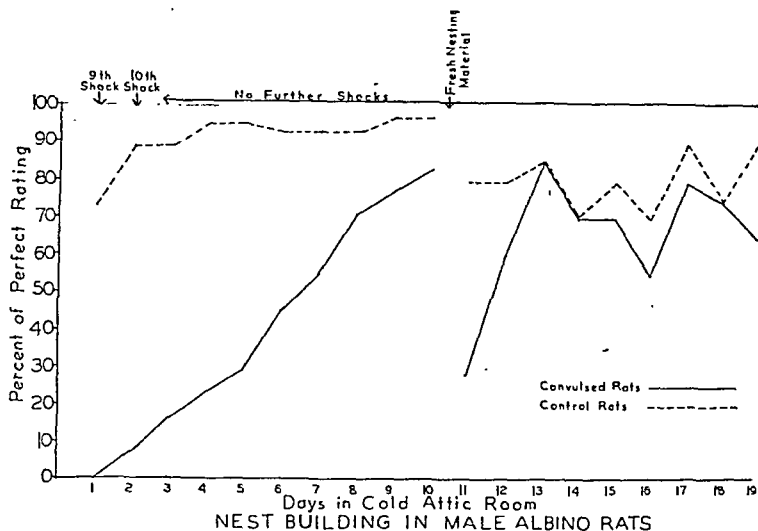


FIG. 1.

Beach<sup>3</sup> has stated that adequate functioning of innately organized reproductive behavior depends on the organism being under the influence of certain hormones at critical periods. Unless the hormones are present in sufficient strength at the right time, stimuli specific to an innately organized pattern of behavior have no effect. Rosvold<sup>4</sup> suggested that the inability of the parturient mother to respond to the paper and shavings as nesting materials may have been due to the fact that the innate organization for these responses had not been sensitized by the appropriate

hormones. He implied that the hormones affected were related to the pregnancy-lactation cycle. Such an hypothesis, without qualification, is no longer tenable.

**Summary.** Twelve adult male albino rats were shocked once a day for 9 days after which they were placed in a relatively cold attic room and supplied with nest building materials. Nine, not shocked but otherwise treated like the others, served as controls. In the early post shock period the convulsed animals made no attempt at nest building whereas their controls built excellent nests. There was gradual recovery so that by the tenth day post shock, the convulsed animals built nests equal to those of the control rats.

<sup>3</sup> Beach, F. A., *Hormones and Behavior*, New York: Paul Hoeber, Inc., 1948, pp. 368.

<sup>4</sup> Rosvold, H. E., *J. Comp. Physiol. Psychol.*, 1949, **42**, 207.

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## Hypotensive Action of Influenza A Virus on Rats.\* (17404)

J. EMERSON KEMPf AND H. T. CHANG.†

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The frequent clinical observations of cardiovascular disturbances complicating influenza

infection<sup>1,2</sup> prompted us to undertake an experimental investigation of the toxic effect

\* This investigation was supported in part by a research grant from the division of research grants and fellowships of the National Institute of Health, United States Public Health Service.

† Research Fellow in Medicine, College of Medicine, University of Illinois.

<sup>1</sup> Findland, M., et al., *Am. J. Med. Sc.*, 1945, **209**, 455.

of the virus on the cardio-vascular system of the host. During a study on the cardiac output of ferrets infected by the PR8 strain of influenza virus<sup>3</sup> arterial hypotension was occasionally noticed. Further observations disclosed that this phenomenon was invariably produced when albino rats were given intravenous injections of the virus. A series of experiments were then carried out to determine the nature of this factor in our preparation.

**Preparation of the virus.** A PR8 strain of influenza virus which was lethal for mice on intravenous injection<sup>4</sup> was originally obtained from Dr. W. Henle and maintained by passages in embryonated eggs by the usual procedures of allantoic sac inoculation, incubation and harvest.<sup>5</sup> The pooled, bacteria-free allantoic fluid was centrifuged at 1050 g to remove the debris and then purified by adsorption and elution from chick red blood cells.<sup>6</sup> The eluate was then centrifuged at 20,000 g for one hour and the precipitate was suspended in 0.1 M phosphate buffer solution of pH 7.0. A final centrifugation at 1050 g removed larger particles. In two experiments, the material was further purified by methanol precipitation according to the method of Cox *et al.*<sup>7</sup> The final product has an average hemagglutination titer<sup>8</sup> of 64,000 per mg of nitrogen, and its infectivity titer for chick embryo was  $10^{13.4}$  g. The extent to which our material was free from non-viral proteins was about the same as the preparations of other authors working on the purification of influenza virus.<sup>9,10</sup>

**Preparation of the animals.** 250-300 g male albino rats (Harlan strain) were anesthetized

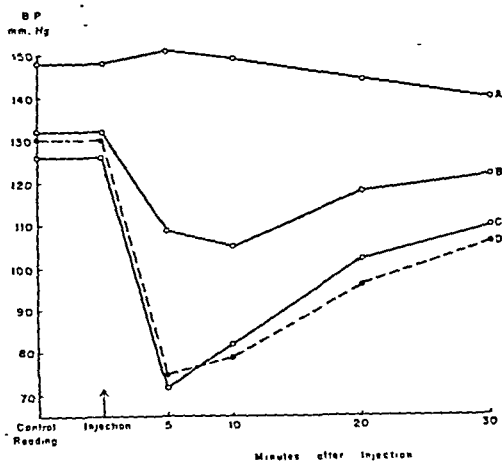


FIG. 1.

Hypotensive action of virus prepared by different purification procedures and that of normal allantoic fluid.

- A—Inj. of conc. normal allantoic fluid.
- B—Inj. of virus conc. by adsorption and elution from chick rbc.
- C—Inj. of virus conc. by adsorption and elution from chick rbc. plus high speed centrifugation.
- D—Inj. of virus conc. by adsorption and elution from chick rbc. plus high speed centrifugation and methanol precipitation.

with sodium nembutal and the trachea was cannulated. Arterial blood pressure was obtained by direct cannulation of the common carotid artery and registered by mercury manometer writing on a kymographic drum. One ml of the virus suspension per 100 g of body weight was injected into the jugular vein at the rate of one ml per minute. The blood pressure was then observed for the next 30 minutes.

**Results.** The data of the experiments can be summarized in the following categories:

1. Allantoic fluid of normal 11-day-old chick embryos was harvested, purified and concentrated by the same procedures used for the virus. Injection of this control material did not alter the blood pressure (Fig. 1, A).

2. The hypotensive factor seemed to be associated with the virus particles since it was similarly adsorbed and eluted from chicken red blood cells sedimented by high speed centrifugation and precipitated by methanol (Fig. 1, B, C, D). When the infectious allantoic fluid was centrifuged at 20,000 g. the precipitate resuspended in buffer solution gave

<sup>2</sup> Andrews, C. L., *J. Med. Soc. N. J.*, 1940, **37**, 16.

<sup>3</sup> Kempf, J. E., and Chang, H. T., submitted for publication.

<sup>4</sup> Henle, G., and Henle, W., *J. Exp. Med.*, 1946, **84**, 623.

<sup>5</sup> *Diagnostic Procedures for Viruses and Rickettsial Diseases*, Am. Pub. Health Assn., 1948, 99.

<sup>6</sup> Hare, R., *et al.*, *Canad. J. Pub. Health*, 1946, **37**, 284.

<sup>7</sup> Cox, H. R., *J. Immunol.*, 1947, **56**, 148.

<sup>8</sup> Salk, J. E., *J. Immunol.*, 1944, **49**, 87.

<sup>9</sup> Sharp, A. R., *J. Immunol.*, 1944, **48**, 129.

<sup>10</sup> Stanley, W. M., *J. Exp. Med.*, 1944, **70**, 255.

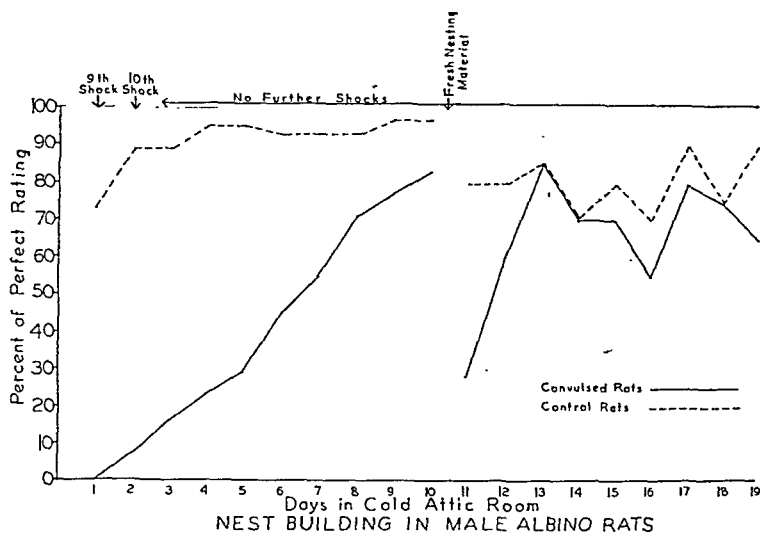


FIG. 1.

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<sup>3</sup> Beach, F. A., *Hormones and Behavior*, New York: Paul Hoeber, Inc., 1948, pp. 368.

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J. EMERSON KEMPf AND H. T. CHANG.†

From the Department of Bacteriology, College of Medicine, University of Illinois, Chicago.

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<sup>1</sup> Findland, M., et al., *Am. J. Med. Sc.*, 1945, **209**, 455.

the loss of the hypotensive factor indicating specific neutralization of the factor (Table II, A).

To prove further that the hypotensive factor was a fraction of the virus particles, the concentrated and purified preparation was mixed directly with the immune serum. Injection of the mixture into rats showed a significant reduction of the hypotensive effect in comparison with the control material to which normal ferret serum was added (Table II, B, C).

6. To elicit the hypotensive response a threshold concentration of virus with hemagglutination titer of 1:8000 was required. Concentrations of virus with titer between 1:16,000 and 1:64,000 elicited about the same response (Fig. 2).

*Conclusion.* These observations indicate that a fraction of the PR8 strain of influenza virus possesses the toxic property of lowering arterial blood pressure in rats.

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### Effect of Congo Red on the "MM" Virus. (17405)

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Conflicting reports on the effect of trypan red on the "MM"<sup>1</sup> neurotropic virus have appeared in the literature. Wood and Rusoff<sup>2</sup> reported this compound along with two other acid dyes, Congo red and brilliant vital red, to be definitely effective against the virus. Using the intraperitoneal injection route followed by intraperitoneal inoculation of the virus, these workers found an apparent protection which was overcome by inoculation of the virus in too high concentration.

Hammon *et al.*<sup>3</sup> have reported the failure of trypan red to protect against the "MM" virus when the dye was injected subcutaneously and the virus inoculated by intraperitoneal route. These authors interpreted the results of Wood and Rusoff as being due to a non specific protection such as afforded by various inert substances when injected previous to the inoculation of infectious agents by the same route.

Since the effect of Congo red had been reported for only one concentration of "MM"

virus it was thought desirable to determine the activity over a range of dilutions.

In one series of experiments Congo red was injected intraperitoneally into 12 g mice and in a second series the dye was administered by stomach tube. As a matter of interest trypan red was included in 2 of the experiments. All virus inoculations were by the intraperitoneal route, serial dilutions being made from frozen stock material. A range (10-fold) of infecting dilutions was employed so as to permit calculation of LD<sub>50</sub> values by the method of Reed and Muench.<sup>4</sup> Unless otherwise specified, dilutions of 10<sup>-6</sup> through 10<sup>-9</sup> were used for controls and 10<sup>-7</sup> through 10<sup>-8</sup> for test groups, 6 mice being used per dilution.

In all experiments calling for the injection of dye the schedule employed was that used by Wood and Rusoff. A 1% solution was injected in 0.1 cc amounts on each of 3 days and the virus was administered one day after the last injection.

For the experiments in which the dyes were given by stomach tube the schedule called for the feeding of 0.5 ml of a 1% solution twice daily for 9 feedings with the virus inoculation following the fourth feed-

<sup>1</sup> Jungeblut, C. W., and Dalldorf, G., *Am. J. Pub. Health*, 1943, 33, 169.

<sup>2</sup> Wood, H. G., and Rusoff, I. I., *J. Exp. Med.*, 1945, 82, 297.

<sup>3</sup> Hammon, W. McD., Aird, R. B., and Sather, Gladys, *Proc. Soc. Exp. Biol. and Med.*, 1948, 69, 511.

<sup>4</sup> Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, 27, 493.

TABLE I.

Effect of Heat on Hemagglutination, Chick Embryo Infectivity, Mouse Toxicity and Hypotensive Factor of the Concentrated Virus Preparation.

Treatment of the purified virus	Hemagglutination titer	Infectivity titer	Mouse toxicity*	% of drop in B.P. after inj. into rats†
Unheated	1:16000	10-10	1:16	42.9
Heated at 56°C for 4 hr	1:8000	10-1	1:4	28.2
8 hr	1:200	0	1:2	33.6
12 hr	0	0	0	18.2
16 hr	0	0	0	5.4‡

\* Kills 4 out of 6 in 48 hr.

Max. drop of B.P. in mm Hg

† % of drop in B.P. =  $\frac{\text{Max. drop of B.P. in mm Hg}}{\text{Control B.P. reading before inj.}} \times 100$ .

Control B.P. reading before inj.

‡ This figure is equivalent to spontaneous variation in B.P. during the period of observation on control animals.

TABLE II.

Effect of Specific Anti-Serum on Neutralization of Hypotensive Factor of the Virus Preparation.

Preparations injected into rats	No. of rats used	Max. drop in B.P. during first 30 min. after inj.*	% drop in B.P. after inj.
A. Infectious allantoic fluid neutralized first and then concentrated	6	0 mm Hg	0
B. Purified virus plus specific immune ferret serum	5	17	11.2
C. Purified virus plus normal ferret serum	6	49	32.9†

\* Initial B. P. for all these animals during control period before injection (average) =  $150 \pm 2$  mm Hg.

† Difference between values for B and C is highly significant statistically. (P value less than 0.01).

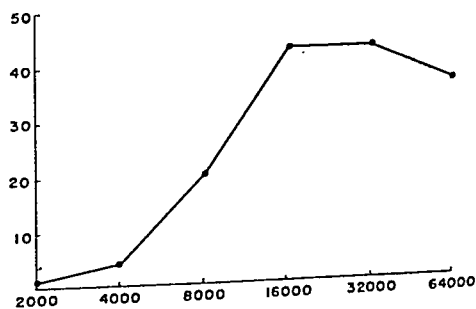
a maximal decline of 40 per cent in blood pressure while the supernatant of the same preparation was entirely without effect.

3. The hypotensive factor was found to be relatively heat resistant being destroyed only after heating at 56° C for 16 hours (Table I). The hemagglutinins, infectivity for chick embryos and toxicity for mice were all inactivated by heating at the same temperature for shorter periods of time respectively.

4. Ferret immune serum titering 1:12500 by the hemagglutination inhibition test was produced by injection of infected mouse or ferret lung tissue suspension. Addition of this serum to pooled allantoic fluid of infected chick embryos resulted in complete neutralization of the virus as indicated by the hemagglutination and chick embryo neutralization tests. This neutralized mixture was then treated by adsorption and elution from chick

red blood cells plus high speed centrifugation. Injection of the resulting preparation showed

% Drop in B.P.  
after injection



Concentration of Virus

FIG. 2.

Relationship between concentration of the virus (shown by hemagglutination titer) and hypotensive response.

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## SECTION MEETINGS

### ILLINOIS

Searle Laboratories

October 18, 1949

### MINNESOTA

Mayo Clinic

October 21, 1949

### PACIFIC COAST

University of California, Davis

September 17, 1949

### WESTERN NEW YORK

University of Buffalo

October 22, 1949

## Influence of Hyaluronidase and Steroids on Permeability of Synovial Membranes. (17495)

JOSEPH SEITZER, DAVID H. BAETTER, AND ALBERT J. BGDANY (Introduced by W. E. Ehrlich)

*From the Wyeth Institute of Applied Biochemistry, Wyeth Incorporated, Philadelphia, Pa.*

It has previously been reported<sup>1</sup> that hyaluronidase reversibly accelerated osmosis and abolished the semipermeable nature of membranes prepared from the excised urinary bladder of rabbits. Skeletal and cardiac muscle membranes were not affected. Adrenal cortical extract and estrone diminished permeability of bladder membranes and antagonized the effect of hyaluronidase. Desoxy corticosterone acetate enhanced the action of hyaluronidase.

The effect of hyaluronidase and steroids on membranes *in vivo* has not been studied heretofore. For this purpose we selected the synovial membrane of rabbits and humans. The synovial membrane and synovial fluid contain hyaluronic acid and are altered in arthritis.<sup>2</sup> The alteration has been attributed to a specific effect on the hyaluronic acid by

hyaluronidases<sup>3</sup> and a structural change in the joint brought about by desoxycorticosterone intoxication as a disease of adaptation.<sup>4</sup> Arthritis has been treated successfully by the administration of Compound E or of adrenocorticotrophic hormone, which presumably releases this compound from the adrenal cortex.<sup>4</sup> It was therefore of interest to determine whether hyaluronidase and desoxycorticosterone weaken the normal synovial barrier by increasing its permeability and whether either adrenal steroids, either administered by injection or released endogenously by the alarm reaction, would strengthen the barrier and antagonize the effects of hyaluronidase.

The experiments on rabbits are reported in this paper. The data obtained on humans under the influence of various drugs and

<sup>1</sup> Seitzer, H. J. *Chm. Experim.* 1948, 6, 117.

<sup>2</sup> Hensch, P. S., Kestell, E. C., Storch, C. H., and Peckay, H. F. *Proc. Staff Meet., Mayo Clinic*, 1949, 24, 151.

<sup>3</sup> Seitzer, J., Baetter, D. H., and Derricks, A. *Proc. Soc. Exp. Biol. and Med.* 1949, 72, 154.

<sup>4</sup> Meyer, R. *Physiol. Rev.* 1947, 27, 335.

TABLE I.  
Effect of Intraperitoneally Injected Dye on "MM" Virus.

	Total No. of mice	Control, LD <sub>50</sub>	Test, LD <sub>50</sub>	LD <sub>50</sub> Difference
Congo red	54	8.2	6.0*	-2.2
	100	7.0†	7.1‡	+0.1
	48	7.5	>5.0	>-2.5
Trypan red	48	7.0	7.7	+0.7

\* Dilution range 10<sup>-4</sup> through 10<sup>-8</sup>.

† Dilution range 10<sup>-5</sup> through 10<sup>-9</sup>, 10 mice per dilution.

‡ Dilution range 10<sup>-4</sup> through 10<sup>-8</sup>, 10 mice per dilution.

TABLE II.  
Effect of Orally Administered Dye on "MM" Virus.

	Total No. of mice	Control, LD <sub>50</sub>	Test, LD <sub>50</sub>	LD <sub>50</sub> Difference
Congo red	48	7.5	5.2	-2.3
	48	8.2	5.8	-2.4
Trypan red	48	7.0	5.6	-1.4

ing. It was felt that two daily feedings would roughly approximate the amounts administered in drug diets.

The results of experiments in which the dye and the virus were administered by intraperitoneal injection are shown in Table I. It can readily be seen that any protection index based on the LD<sub>50</sub> differences recorded for Congo red will have a wide range and the validity of a claim for protective action would be open to serious question. The erratic results presented are typical of other experiments and it is not felt that a truly protective compound would be subject to such variable results.

Table II shows the effect of dye administered orally, the virus being given by intraperitoneal injection. While the apparent protection may be due to non specific factors, such as handling of the mice, it is of interest to note that such apparent protection may be

evoked by administration of the dye through a route other than that used for the virus injection.

Calculation of the LD<sub>50</sub> standard errors would undoubtedly show that little confidence could be placed in the LD<sub>50</sub> differences recorded and it is not felt that any of the differences are indicative of significant protection. In considering the work of Wood and Rusoff it would not seem that a really effective compound would be active against one concentration of virus only and we feel that the expression "apparent protection" is more accurate.

*Conclusions.* Congo red was found to stimulate an apparent protection against the "MM" virus when administered by a route other than that used for virus inoculation. It is not felt that the degree of protection is significant.

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TABLE I.  
Influence of Hyaluronidase and Steroids on Permeability of Synovial Membrane *in vivo*.

Procedure	No. of rabbits	Min. from instillation of PSP i.j. to its appearance in urine				Gamma PSP in 1 cc of urine					
		First		Dur'n of excretion		Onset		Peak		End	
		Avg	Range	Peak	Avg	Range	Avg	Range	Avg	Range	
Control	16	14	13-16	30	45	43-48	49	48-50	126	123-127	3 3
150 TRU Hyalase* i.j.	6	9	8-10	9	20	19-21	119	118-119	119	118-119	4 4
12,000 TRU Hyalase/kg i.v.	2	12	12-13	30	46	46-47	52	52	102	102	4 4
120,000 TRU Hyalase/kg i.v.	1	8	—	8	25	—	110	—	110	—	7 —
U.S.F. (non-Hyalase)	2	14½	14-15	30	47	—	48	48-49	126	125-127	35 3-4
1 u. A.C.E. i.j.	3	23	23-24	30	86	86-87	30	30-31	68	68	49 49-50 (60 min.)
100 u. A.C.E. i.m.	4	22	19-22	30	139	138-140	40	40-41	49	49	21 21 (130 min.)
1 u. A.C.E. i.j. + Hyalase i.j.	3	20	19-20	30	73	72-73	42	42	54	54-55	41 41 (70 min.)
100 u. A.C.E. i.m. + Hyalase i.j.	4	17	16-18	30	135	135-136	46	46-47	58	58-59	25 25 (130 min.)
5 mg DOCA i.m.	2	10	10	10	19	19-20	112	112-113	112	112-113	4 4
5 mg DOCA i.m. + Hyalase i.j.	2	9	9-10	9	19	19-20	111	111	111	111	5 5
Alarm reaction†	2				More than 2¼ hr				No dye in urine in 2¼ hr		
									All present in joint cavity at autopsy		
Acute adrenalectomy	2	14½	14-15		46	46			Specimens contaminated by hematuria		4
Acute adrenalectomy + Hyalase i.j.	1	10	—	10	21	—	118	—	118	—	—
2 mg Estrone i.m.	2	15	15	15	243	242-244	69	69-70	69	69-70	4 4
2 mg Estrone i.m. + Hyalase i.j.	2	19	19-20	19	246	246	70	70	70	70	5 5

\* Testicular hyaluronidase, Wyeth.

A.C.E. = Adrenal cortical extr. (alcoholic), Upjohn Co.

DOCA = Desoxycorticosterone acetate in oil.

† Induced by 3 mg Colchicine/kg body wt subcutaneously.

i.j. = In tubercular articulation.  
TRU = Turbidity reducing units.  
i.m. = Intramuscular inj.  
PSP = Phenolsulphonphthalein.  
USF = Urinary spreading factor.



diseases will be reported in a subsequent paper. by Fitch, D.R., Corn, O., Jallo, Jr., S. J., and Seifter, J.

*Material and methods.* Sixteen male white rabbits weighing between 3.5 and 5.0 kg were prepared for this study by a modification of the method reported by Curtis and Brunschwig for studying the human knee joint.<sup>5</sup> Each animal was anesthetized with 40 mg sodium pentobarbital per kg intraperitoneally, then placed on its dorsal surface and the inner aspect of each right hind leg was shaved at the talocrural articulation. A soft rubber catheter was inserted through the urethra into the bladder and retained there for the duration of the experiment. A sample of urine was removed immediately to show that no dye was present in the urine at the beginning of the experiment. Then by inserting a 26 gauge one-inch needle from the inner aspect of the ankle, 1.25 mg phenolsulphonphthalein (PSP) in 0.25 ml of 0.85% NaCl solution was injected into the cavity of the talocrural articulation. After insertion of the needle the syringe was aspirated to insure the presence of the needle in the joint cavity without having caused trauma and to check against the rupture of any small blood vessels. The joint was kept extended at all times throughout the experiment. Every 5 minutes after injection the urine was tested for the presence of dye. At its first appearance and every 15 minutes thereafter the amount of dye in 5 ml urine was measured in a Klett colorimeter by the method of Dandy and Rowntree.<sup>6</sup> If its disappearance from the urine was rapid, measurements were taken more often. The bladder was emptied after each sample of urine was withdrawn for a reading. All animals were standardized in the manner just described from one to 15 days before any other compound was administered. All instillations were made into the right hind talocrural articulation. The maximum number of tests in each rabbit was 5.

The doses and channel of administration

<sup>5</sup> Curtis, G. M., and Brunschwig, A., *Proc. Soc. Exp. Biol. and Med.*, 1930, **27**, 358.

<sup>6</sup> Dandy, W. E., and Rowntree, L. G., *Ann. Surg.*, 1914, **59**, 587.

of each compound used are listed in Table I. Compounds injected directly into the joint cavity were administered along with the dye. All intramuscular and intravenous injections were made 30 to 90 minutes before PSP or hyaluronidase was instilled into the joint cavity. The hyaluronidase used was prepared from bull testes and assayed 8400 turbidity reducing units per mg of nitrogen. The urinary spreading factor was isolated in our laboratory from normal urine and has been shown not to be hyaluronidase. Ten mg of the urinary spreading factor had the same activity as 150 turbidity reducing units of hyaluronidase in causing the spread of dye in the skin of rabbits. Colchicine was administered as the alarming stimulus to 2 rabbits in order to cause endogenous release of adrenal corticoids.

*Results.* The effects of hyaluronidase and steroids on the permeability of the synovial membrane are shown in Table I. Dye first appeared in the urine of the control animals on an average of 14 minutes after instillation of PSP into the synovial cavity. The urinary concentration of dye at this time was 49  $\gamma$  per ml. The peak concentration was reached within 30 minutes with an average of 126  $\gamma$  per ml. Excretion was complete on an average of 40 minutes, at which time the concentration was 3  $\gamma$  per ml. The ranges of variation for the time of initial appearance and for the initial concentration were surprisingly small. The time to reach the peak, the concentration at 30 minutes, and the duration of excretion showed similar constancy.

1. *Effect of hyaluronidase and of urinary spreading factor.* Instillation of 150 turbidity reducing units of hyaluronidase and PSP into the joint resulted in more rapid absorption and elimination of the dye. The first appearance of dye was in 9 minutes, with a urinary concentration of 119  $\gamma$  per ml, which was the peak. The duration of excretion was 20 minutes. Intravenous injection of 120,000 turbidity reducing units of hyaluronidase per kg of body weight caused the dye to first appear in the urine in 8 minutes at a concentration of 110  $\gamma$  per ml, which was the peak. Excretion was completed in 25 minutes. Intravenous injection of 12,000 turbidity reduc-

state of permeability of synovial membranes could be governed by the balance between DOCA and some other adrenal steroid. A similar opposition of effect was noted in isolated membranes<sup>1</sup> and in human hypertensives.<sup>9</sup> Furthermore, DOCA aggravates rheumatoid arthritis,<sup>10</sup> while Compound E alleviates this disease.<sup>4</sup>

The synovial membrane of rabbits in the alarm reaction phase of the adaptation syndrome was completely impermeable to PSP. The dye could be recovered from the joint more than 2 hours after its instillation. Isolated urinary bladder membranes prepared from rabbits in the alarm reaction are impermeable to the passage of fluid when used to separate M/1 sucrose from distilled water, while such bladders prepared from normal rabbits act as semipermeable membranes under the same conditions.<sup>1</sup> Such semipermeable membranes become impermeable to the further passage of fluid if serum from a rabbit in the alarm reaction is added to them.<sup>1</sup>

Adrenal steroids are released during the adaptation syndrome in response to physical, chemical, and psychic stresses. A protective role is attributed to these in the alarm reaction stage.<sup>3,11</sup> Their role during the stage of adaptation is not clear, but the diseases that are thought to arise during this stage are attributed to DOCA intoxication. It might well be that protective steroids possibly of the Compound E type are predominant during the alarm reaction phase and that possibly DOCA types dominate the adaptation phase. Alleviation of adaptation disease could then be obtained by administration of Compound E types or by the administration of adrenocorticotrophic hormone. It is of interest in this respect that DOCA does not affect the blood pressure of normotensive individuals but aggravates hypertension, which is considered to be a disease of adaptation,<sup>9</sup> and that the aggravation is nullified by ACE.

The synovial membrane permeability was

not altered shortly after adrenalectomy, nor was the response to hyaluronidase abnormal. Depletion of adrenal steroids could not have occurred in this short period. Changes in permeability will probably be seen in the animals under study at various intervals after adrenalectomy.

The findings reported in this paper may be of particular significance in considering the pathogenesis and treatment of rheumatoid arthritis. This disease appears to be a deficiency in the function of hyaluronic acid of the synoviae. We have demonstrated that similar abnormal function of lowered resistance and increased permeability can be induced by either hyaluronidase or DOCA, both of which could permit exudation into joints. The etiology of rheumatoid arthritis has been attributed to hyaluronidases attacking the synovial structures<sup>2</sup> and to what may be DOCA intoxication due to various stresses.<sup>6</sup> We have furthermore shown that the effect of hyaluronidase is antagonized by ACE and endogenously released adrenal steroids by increasing the resistance of the synovial barrier. We have also demonstrated that DOCA acts in opposition to the other steroids present in ACE. These findings are to be considered in explaining the mechanism of the beneficial effect of Compound E, adrenocorticotrophic hormone, and possibly colchicine and salicylates in the treatment of rheumatoid arthritis and rheumatic fever.<sup>12,13</sup>

**Summary and conclusions.** 1. The permeability of the synovial membrane as measured by speed of absorption and excretion into the urine of phenolsulphonphthalein (PSP) instilled into the joint was found to be surprisingly constant in a group of 16 normal rabbits.

2. Hyaluronidase markedly increased permeability of the synovial membrane. The effect was maximal and was not augmented by desoxycorticosterone acetate (DOCA).

3. Adrenal cortical extract decreased permeability of the synovial membrane and an-

<sup>9</sup> Perera, G. A., and Pines, K. L., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 443.

<sup>10</sup> Degannes, L., Mahoudeau, D., and Bricaire, H., *Bull. Soc. Med. Hosp. Paris*, 1947, **63**, 532.

<sup>11</sup> Long, C. N. H., *Fed. Proc.*, 1947, **6**, 461.

<sup>12</sup> Forman, C., Seifter, J., and Ehrlich, W. E., *J. Allergy*, 1949, **20**, 273.

<sup>13</sup> Hench, P. S., Slocumb, C. H., Barnes, A. R., Smith, H. L., Polley, H. F., and Kendall, E. C., *Proc. Staff Meet., Mayo Clinic*, 1949, **24**, 277.

ing units of hyaluronidase per kg resulted in no significant change from the normal excretion pattern.

Instillation of urinary spreading factor in an amount equal to the spreading activity of 150 turbidity reducing units of hyaluronidase did not alter the normal excretion pattern.

2. *Effect of adrenal cortical extract (ACE).* Instillation of one unit of ACE into the joint or intramuscular injection of 100 units delayed the initial appearance of dye in the urine to 23 minutes at a concentration of 30 to 40  $\gamma$  per ml. The peak of 49 to 68  $\gamma$  per ml was attained within 30 minutes. Excretion was prolonged so that the concentration at 60 minutes was 49  $\gamma$  per ml and 21  $\gamma$  in 130 minutes. Instillation of 150 turbidity reducing units of hyaluronidase into the joint of ACE treated rabbits did not significantly alter the excretion pattern obtained with either channel of administration of ACE.

3. *Effect of desoxycorticosterone acetate (DOCA).* Intramuscular injection of DOCA decreased the time for initial appearance of dye in the urine to 10 minutes at a concentration of 112 gammas per ml, which was also the peak. Excretion was completed in 19 to 20 minutes. Instillation of 150 turbidity reducing units of hyaluronidase into the synovial cavity of DOCA treated rabbits did not further hasten absorption and excretion.

4. *Effect of alarm reaction.* PSP was not absorbed from the synovial cavity of rabbits in colchicine alarm reaction, and all the dye could be recovered from the synovial cavity more than two hours after its instillation.

5. *Effect of adrenalectomy.* The pattern of excretion begun 30 minutes after adrenalectomy was essentially the same as in the intact controls. Hyaluronidase had no greater effect in such an adrenalectomized rabbit than in intact rabbits.

6. *Effect of estrone.* Dye first appeared in the urine of estrone treated rabbits in 15 minutes at a concentration of 69 gammas per ml, which was the peak. The duration of excretion was prolonged to 243 minutes. Instillation of 150 turbidity reducing units of hyaluronidase into the joint of estrone treated rabbits did not significantly alter this pattern.

*Discussion.* The surprising uniformity of

rate of absorption from the joints of normal rabbits and the constant effect of hyaluronidase lends great weight to the results obtained when small groups of animals were subjected to the other experimental procedures under test and when small changes were obtained. The more rapid absorption of PSP from the joint of rabbits receiving hyaluronidase is undoubtedly due to increased permeability of the synovial membrane. Presumably this is a specific effect on the hyaluronic acid of this membrane since a non-hyaluronidase spreading factor did not alter its permeability. The large amount of hyaluronidase that was needed by intravenous injection to produce the same effect as a small amount applied locally is accounted for by the presence of hyaluronidase inhibitors in the blood.

The delayed and prolonged absorption of PSP from the joint of rabbits receiving adrenal cortical extract is undoubtedly due to decreased permeability of the synovial membrane. ACE probably altered the state of polymerization of the hyaluronic acid of the membrane since hyaluronidase was no longer effective in increasing its permeability in ACE treated animals. Inhibition of hyaluronidase activity by ACE has been observed in skin<sup>7</sup> and isolated membranes.<sup>1</sup> Estrone treatment also prolonged but did not significantly delay the onset of absorption of PSP. Since hyaluronidase did not increase the permeability of the synovial membrane in the estrone treated rabbits, it is assumed that estrone also altered the hyaluronic acid of the membrane. Increased resistance to hyaluronidase by estrone has been observed in skin<sup>8</sup> and isolated membranes.<sup>1</sup>

The synovial membrane was more permeable to PSP during DOCA treatment than in the control period. The effect was identical with that produced by hyaluronidase. It was maximal and could not be augmented by the instillation of hyaluronidase into the joint. It is significant that the total adrenal steroids had the opposite effect of one of them. The

<sup>7</sup> Opsahl, J., White, A., and Duran-Reynals, F., *Ann. N. Y. Acad. Sci.*, in press.

<sup>8</sup> Sprunt, D. H., and McDearman, S., *Endocrinology*, 1940, **27**, 893.

5 animals were autopsied 6 months after the institution of hormone treatment. Administration of hormone was suspended 53 days before autopsy in 2 NH females which received similar treatment.

Two ovariectomized NH females received the same treatment with the estrogen and in addition were injected weekly with 150  $\mu$ g of progesterone in oil. Two additional ovariectomized females received the estrogen plus progesterone, but treatment was suspended 47 days before autopsy.

Two NH females ovariectomized at 21 days of age were given 17.5  $\mu$ g of testosterone propionate in oil weekly by subcutaneous injection beginning 2 weeks after operation. This treatment was continued until the animals were autopsied 5½ months after ovariectomy. Three mice were similarly treated except that they received no injections of hormone for the 46 days preceding autopsy.

Histologic serial sections of the adrenal glands were studied following complete autopsy. Representative sections of the reproductive tracts, submaxillary glands and kidneys were observed to assay the hormonal status of the experimental animals.

Cortical adenomas appeared in gonadectomized mice of the NH strain receiving amounts of estrogenic hormone just sufficient to maintain constant estrus. Ovariectomized females receiving masculinizing doses of testosterone propionate (as judged by submaxillary gland

histology) also developed cortical adenomas. Similar adenomas were present in the ovariectomized females which received estrogen and progesterone simultaneously, and in ovariectomized females receiving no hormone injections. A group of 4 ovariectomized NH females which received 0.1  $\mu$ g of estradiol dipropionate weekly for 4 months following operation at 35 days of age had definite adenomas at autopsy.

Seventeen of 20 gonadectomized mice on hormone treatment developed adenomas which were in all respects similar to those observed in controls which had been merely gonadectomized. Weekly injections of 0.05  $\mu$ g of estradiol dipropionate failed to prevent the development of spontaneous adrenal cortical adenomas in NH females.<sup>3</sup>

Since adrenal cortical adenomas developed in gonadectomized mice receiving maintenance doses of sex hormone, it appears that absence of functional gonadal tissue, rather than withdrawal of sex hormone, is the prime factor in the development of adrenal cortical adenomas in gonadectomized mice. The gonad, as compared to the adrenal, may preferentially utilize trophic hormone of the pituitary gland. When functional gonadal tissue is absent in a strain of mice with an extragonadal sensitive target organ, such as the NH adrenal cortex, adenomas develop.

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<sup>3</sup> Frantz, M. F., unpublished data.

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## Search for Antibiotics Active Against *Mycobacterium tuberculosis*. 1. A Streptothricin-like Antibiotic from a *Streptomyces* sp.\* (17408)

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In a search for antibiotics active against *Mycobacterium tuberculosis* var. *hominis* several hundred antagonists have been encountered and one called *Streptomyces* sp. EI<sub>2</sub> in our records was chosen for intensive study because of its marked activity against the mycobacteria and the low animal toxicity

of its crude filtrate.<sup>1</sup> The present report is

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tagonized the effect of hyaluronidase. This effect of adrenal steroids was more pronounced when they were released endogenously by the alarm reaction. Estrone also decreased the permeability of synovial membrane.

4. Desoxycorticosterone increased maximally the permeability of synovial membrane to the same extent as hyaluronidase and could not be augmented by hyaluronidase.

5. It is suggested that the normal permeability of the synovial membrane is in part controlled by the balance between adrenal steroids of the DOCA type and of the Compound E type.

6. These findings are consistent with current views of the etiology of rheumatoid arthritis either by hyaluronidases or from exposure to stressing stimuli. They are also consistent with the therapeutic effects of Compound E

or of adrenocorticotrophic hormone in the treatment of arthritis.

*Addendum:* Since completion of this work cortisone acetate and adrenocorticotrophic hormone (ACTH) were made available to us. One mg of cortisone acetate per kg and 0.5 mg of ACTH per kg were almost as effective in decreasing permeability of the synovial membrane as the alarm reaction. With both compounds only 25 to 30% of the dye was excreted in 4½ hours. The remainder was recovered from the synovial cavity. On this basis, the dose of adrenal cortical extract and estrone listed in Table I has cortisone activity of less than 20%, and the normal membrane balance of DOCA:cortisone an activity of 4.5%.

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### Development of Adrenal Cortical Adenomas in Ovariectomized Mice Injected with "Physiologic" Doses of Sex Hormone.\* (17407)

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Since the development of adrenal cortical adenomas in gonadectomized mice has been inhibited by the implantation of pellets of estrogenic hormone,<sup>1</sup> it might be inferred that withdrawal of gonadal secretion is of primary importance in precipitating the development of the castration-induced adenoma of the mouse adrenal cortex. The objective of the experiments being reported was to determine the effect of the administration of regulated, "physiologic" amounts of estrogenic or androgenic hormone on the induction of adrenal

cortical tumors in susceptible ovariectomized mice.

Mice of the NH strain were used in these experiments. NH females develop adrenal cortical tumors spontaneously following ovarian regression, and precociously if gonadectomized.<sup>2</sup> Adrenal cortical adenomas are present in most NH mice of both sexes 5 months after removal of the gonads.

Four NH males and one female which had been gonadectomized at 23 days of age received (by subcutaneous injection beginning 2 weeks after operation) 0.05 microgram of estradiol dipropionate‡ in sesame oil at intervals of from 5 to 9 days. This treatment with estrogenic hormone provided for constant estrus, as determined by vaginal smear. These

\* This investigation has been aided by grants from the National Cancer Institute, The Jane Coffin Childs Memorial Fund for Medical Research, and the Cancer Fund of the Graduate School of the University of Minnesota.

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<sup>1</sup> Woolley, G., and Little, C. C., *Cancer Research*, 1946, 6, 491.

<sup>2</sup> Kirschbaum, A., Frantz, M. F., and Williams, W. L., *Cancer Research*, 1946, 6, 707.

‡ The synthetic sex hormones were supplied by Ciba Pharmaceutical Products through the courtesy of F. E. Houghton.

workers<sup>10</sup> showed that most of the antibiotic was adsorbed on the Decalso column and all dark pigment eliminated.

The eluate was evaporated *in vacuo* to a small volume and the antibiotic brought down as a fine white precipitate by addition of acetone. Still further purification was carried out by the chromatographic method as employed by Carter and co-workers<sup>11</sup> for the purification of streptomycin.

The material was finally concentrated *in vacuo*, reprecipitated with acetone, dissolved in double glass-distilled water and sterilized by passage through a sintered glass filter.

**D. Methods of Assaying EI<sub>5</sub>.** The potency of EI<sub>5</sub> was determined by a serial dilution method employing the broth base of the medium recommended by Loo and co-workers<sup>10</sup> for streptomycin assay. The unitage is expressed as dilution units using *Escherichia coli-communior* as the test organism. On the basis of dry weight, the final product was bacteriostatic for *E. coli-communior* in a dilution of approximately 1:200,000. The activity of various batches of antibiotic was observed to vary considerably and no consistent results were obtained which would make possible an accurate comparison of the effectiveness of the several purification methods used.

**E. Antibacterial Spectrum of EI<sub>5</sub>.** Antibacterial tests readily established that EI<sub>5</sub> and streptomycin are different antibiotics. For example, the minimum concentrations inhibitive for *Bacillus subtilis* were EI<sub>5</sub>, 1.6 units and streptomycin, 4.0 units. With *Serratia marcescens* the values were EI<sub>5</sub> 30.0 units and streptomycin, 0.75 unit.

The differences between the antibacterial spectra of EI<sub>5</sub> and streptothricin were less marked.

In a further attempt to identify EI<sub>5</sub> resort was made to the development of strains of test organisms resistant to streptothricin and to EI<sub>5</sub>. The parent strains of organisms used

for this work were *B. subtilis*, *E. coli* M3G and *Micrococcus pyogenes* var. *aureus* 209-P. They were cultured at 37°C in nutrient broth pH 7.1, containing varying amounts of antibiotic covering the ranges of tolerance and subcultures made at 24-hour intervals with inocula from the highest concentration of antibiotic showing growth in the previous series.

Sixty or more serial transfers were made over a period of 3 months before cultures of appreciable resistance were obtained (cf. Kelner and Morton,<sup>4</sup> and Trussell, Fulton, and Grant<sup>3</sup>). It was noted that some of the organisms tended to lose their acquired resistance upon repeated subculture on nutrient agar.

The antibacterial spectra of streptothricin and purified EI<sub>5</sub>, determined with the parent and resistant strains shown in Table I, clearly indicate that streptothricin and EI<sub>5</sub> are different substances. It is noteworthy that all of the resistant strains developed against EI<sub>5</sub> also showed marked resistance to streptothricin, whereas, resistant strains developed against streptothricin do not show as uniform and marked increase in resistance to EI<sub>5</sub>. Of additional interest is the case of *B. subtilis* cultivated in EI<sub>5</sub> in which a marked resistance to streptothricin resulted without an increase in resistance to EI<sub>5</sub>.

One explanation which may account for these findings is that the development of resistance to one antibiotic may result in the development of resistance to another closely related antibiotic (cf. Kelner and Morton<sup>4</sup>). Another possible explanation is that the preparation of EI<sub>5</sub> may contain a small amount of streptothricin as an impurity.

**F. Comparison of EI<sub>5</sub> with other Streptothricin-like Antibiotics.** Since lavendulin and actinorubin (Kelner and Morton<sup>4</sup>) were not available, resort was made to cross-streak antibacterial spectral analysis of *S. sp.* EI<sub>5</sub>, *S. sp.* A-10, and *S. sp.* A-105 in an attempt to obtain information on the identity of the respective antibiotics. The results are presented in Table II.

The marked variation in the zones of inhibition shown by *S. sp.* A-10 and *S. sp.* EI<sub>5</sub> indicates strongly that the antibiotic produced

<sup>10</sup> Loo, Y. H., Skell, P. S., Thornberry, H. H., Ehrlich, J., McGuire, J. M., Savage, G. M., and Sylvestre, J. C., *J. Bact.*, 1945, 50, 701.

<sup>11</sup> Carter, H. E., Clark, R. K., Jr., Dickmans, S. R., Loo, Y. H., Skell, P. S., and Strong, W. A., *J. Biol. Chem.*, 1945, 160, 337.

the account of a study of this organism and the antibiotic it produces.

**Methods and Results.** *A. Morphological and Cultural Characteristics of Streptomyces sp. EI<sub>5</sub>.* Since preliminary tests of solubility and antibacterial activity indicated that the antibiotic produced by *S. sp. EI<sub>5</sub>* is similar to streptomycin and streptothricin, a comparison of the organisms that produce these antibiotics was made.

*Streptomyces sp. EI<sub>5</sub>* was morphologically similar to *Streptomyces griseus*, and both organisms produced a soluble yellow to yellow-brown pigment when grown at room temperature on nutrient agar, glucose agar, starch agar, and gelatin. Only *S. griseus* produced pigment on potato agar. The color of the aerial mycelium varied from white to tea-green with *S. griseus* and from white to gray with *S. sp. EI<sub>5</sub>*. The vegetative mycelium of *S. griseus* was white to cream colored and that of *S. sp. EI<sub>5</sub>* yellow to tannish orange. *Streptomyces sp. EI<sub>5</sub>* was not susceptible to the *S. griseus* actinophages of Reilly, Harris and Waksman.<sup>2</sup> These observations indicate that *S. griseus* and *S. sp. EI<sub>5</sub>* are similar but significantly different organisms.

*Streptomyces lavendulae* produced a darker brown pigment on glucose agar, nutrient agar and glucose broth than *S. sp. EI<sub>5</sub>*, and in addition produced pigment on the potato plug. Further, slide cultures on Czapek's medium at 30°C showed the aerial mycelium of *S. lavendulae* to be pink to lavender, and the hyphae often coiled terminally, in contrast to the white to gray color and the straight hyphae of *S. sp. EI<sub>5</sub>*. The conidia of *S. sp. EI<sub>5</sub>* were observed to be oval to globose and considerably smaller than those of *S. lavendulae*. These differences indicate that *S. lavendulae* and *S. sp. EI<sub>5</sub>* are not identical.

It is also probable that *S. sp. EI<sub>5</sub>* is distinct from the organism described by Trussell, Fulton and Grant<sup>3</sup> since, unlike our organism, their organism produces streptomycin and dis-

plays tightly coiled terminal hyphae. In the latter respect, our organism differs also from the two *Actinomyces sp.* described by Kelner and Morton<sup>4</sup> (A-10 and A-105) which produce the antibiotics, lavendulin and actinorubin.

**B. Production of Antibiotic EI<sub>5</sub>.**<sup>†</sup> The 1% glucose medium of Waksman and Woodruff<sup>5</sup> proved to be as satisfactory as any medium tried including modifications of the soybean medium described by Rake and Donovick.<sup>6</sup>

Attempts to enhance antibiotic production by the procedure employed for *S. griseus* by Waksman, Reilly, and Johnstone<sup>7</sup> of growing the organism in high concentrations of its own antibiotic failed. The standard inoculum adopted was a heavy spore suspension of a high antibiotic-producing substrain. The organisms were grown in shake cultures for 5-7 days and the culture fluid harvested when the pH rose to 7.0 to 7.4. It was filtered through cotton and stored at -45°C prior to extraction.

**C. Concentration and Purification of EI<sub>5</sub>.** Since the antibiotic was found to belong to the 3rd solubility group of Waksman,<sup>8</sup> the general scheme of the procedure used by Le Page and Campbell<sup>9</sup> for the concentration of streptomycin was adopted. It consisted of adsorption on carbon, elution with acid methanol and precipitation with acetone. The total yield in this concentrate was approximately 30% of the amount present in the original filtrate.

Further purification was accomplished by the use of the synthetic zeolite Decalso. Spot plate analyses by the method of Loo and co-

<sup>4</sup> Kelner, A., and Morton, H. E., *J. Bact.*, 1947, **53**, 695.

<sup>†</sup> Antibiotic EI<sub>5</sub> is subsequently designated as EI<sub>5</sub>.

<sup>5</sup> Waksman, S. A., and Woodruff, H. B., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 207.

<sup>6</sup> Rake, G., and Donovick, R., *J. Bact.*, 1946, **52**, 223.

<sup>7</sup> Waksman, S. A., Reilly, H. C., and Johnstone, D. C., *J. Bact.*, 1946, **52**, 393.

<sup>8</sup> Waksman, S. A., *Microbial Antagonisms and Antibiotic Substances*, p. 171, The Commonwealth Fund, New York, N. Y., 1945.

<sup>9</sup> Le Page, G. A., and Campbell, E., *J. Biol. Chem.*, 1946, **162**, 163.

<sup>1</sup> Gardner, G. M., Master's Thesis, University of Washington, 1946.

<sup>2</sup> Reilly, H. C., Harris, D. A., and Waksman, S. A., *J. Bact.*, 1947, **54**, 451.

<sup>3</sup> Trussell, P. C., Fulton, C. O., and Grant, G. A., *J. Bact.*, 1947, **53**, 769.

to the 19th day. The results demonstrated that EI<sub>5</sub> is apparently less toxic than streptothricin as judged by the findings of Molitor,<sup>13</sup> and more nearly equal to the toxicity of actinorubin as determined by Morton.<sup>14</sup> Both actinorubin and EI<sub>5</sub> in large dosage cause focal necrosis of the liver and renal epithelial damage.

**Summary.** Search for antibiotics active against *Mycobacterium tuberculosis* var. *hominis* has resulted in the isolation of a streptothricin-like antibiotic, termed EI<sub>5</sub>, from an unidentified *Streptomyces* sp. It is bacteriostatic for *Mycobacterium tuberculosis* H37Rv in dilution of 1:100,000 and closely resembles

<sup>13</sup> Molitor, H., *Ann. N. Y. Acad. Sci.*, 1946, **48**, 101.

<sup>14</sup> Morton, H. E., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 327.

actinorubin in its antibacterial spectrum, general properties, and toxicity.

The organisms that produce the two antibiotics, *Streptomyces* sp. A-105 and *Streptomyces* sp. EI<sub>5</sub> are morphologically distinct.

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## Multiplicity of Antigens in Ragweed Pollen Extracts as Demonstrated by the Technic of Oudin. (17409)

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The heterogeneity of ragweed pollen extracts has been demonstrated by chemical fractionation<sup>1</sup> and by electrophoretic analysis.<sup>2</sup> Precipitins to crude extracts of ragweed pollen have been produced by numerous workers,<sup>3,4</sup> but because of the heterogeneity of the extracts used, it would be expected that the precipitins so obtained were directed to a number of different antigenic substances. It is the purpose of this paper to show, by a new technic, the presence of a multiplicity of antibodies in the antisera produced against crude extracts of ragweed pollen, and consequently, the presence of a multiplicity of antigens in

the extracts used for immunization.

The new technic used to demonstrate this antigenic complexity was that recently described by Oudin<sup>5-9</sup> which consists in overlaying a solid immune serum-agar mixture contained in a thin-bore tube with the antigen solution. An antigen-antibody precipitate is formed as a sharp band which moves down the tube as more and more antigen diffuses into the agar. Oudin showed that only one band is formed when a single antigen-antibody system is present, while multiple systems give

<sup>5</sup> Oudin, J., *Compt. Rend. Acad. Sci.*, 1946, **222**, 115.

<sup>6</sup> Oudin, J., *Bull. Soc. Chim. Biol.*, 1947, **29**, 140.

<sup>7</sup> Oudin, J., *Annal. de l'Inst. Pasteur*, 1948, **75**, 30.

<sup>8</sup> Oudin, J., *Annal. de l'Inst. Pasteur*, 1948, **75**, 109.

<sup>9</sup> Oudin, J., *Compt. Rend. Acad. Sci.*, 1949, **228**, 1900.

<sup>1</sup> Stull, A., Sherman, William, and Wilson, W., *J. Allergy*, 1942, **13**, 537.

<sup>2</sup> Abramson, H. A., *Ann. Allergy*, 1947, **5**, 19.

<sup>3</sup> Kukla, A., and Hirsch, D., *J. Immunol.*, 1946, **53**, 391.

<sup>4</sup> Eagle, H., Arbesman, C. E., and Winkenwerder, W. L., *J. Immunol.*, 1939, **30**, 425.



TABLE I.  
Antibacterial Spectra of Streptothricin and EI<sub>5</sub> Determined with Resistant and Parent Strains of Bacteria.  
(Readings taken after 24 hr at 37°C in nutrient broth at pH 7.0).

Test organism	Min. inhibiting conc. of streptothricin in dilution units	Min. inhibiting conc. of EI <sub>5</sub> in dilution units
<i>Bacillus subtilis</i> (parent)	12	4
" " (streptothricin resistant)	120	8
" " (EI <sub>5</sub> resistant)	90	4
<i>Escherichia coli</i> (parent)	0.6	0.8
" " (streptothricin resistant)	8	8
" " (EI <sub>5</sub> resistant)	20	20
<i>Micrococcus aureus</i> (parent)	0.3	0.5
" " (streptothricin resistant)	22	4
" " (EI <sub>5</sub> resistant)	36	40

TABLE II.  
Cross-streak of Antibacterial Spectra of *Streptomyces* sp. A-10, *Streptomyces* sp. EI<sub>5</sub>, and *Streptomyces* sp. A-105.

Test organism	Zone of inhibition in mm		
	<i>S. sp.</i> A-10	<i>S. sp.</i> EI <sub>5</sub>	<i>S. sp.</i> A-105
<i>Achromobacter</i> sp.	18	16	12
<i>Acrobacter aerogenes</i>	20	30	25
<i>Bacillus cereus</i>	10	3	5
" <i>megatherium</i>	17	15	10
" <i>mycoides</i>	25	30	30
" <i>sp.</i> 290	22	27	20
" <i>subtilis</i>	8	1	3
<i>Corynebacterium xerose</i>	12	2	7
<i>Escherichia coli-communior</i>	20	20	15
<i>Micrococcus aureus</i> 209-P	18	15	15
<i>Sarcina lutea</i>	20	15	10
<i>Serratia marcescens</i>	12	2	2
EI <sub>5</sub> resistant			
<i>Bacillus subtilis</i>	8	5	5
<i>Micrococcus aureus</i>	10	3	5
Streptothricin resistant			
<i>Bacillus subtilis</i>	5	1	1
<i>Escherichia coli-communior</i>	15	7	7
<i>Micrococcus aureus</i>	25	7	12

by *S. sp.* A-10 (lavendulin) is different from EI<sub>5</sub>. In contrast, the zones of inhibition shown by *S. sp.* A-105 and *S. sp.* EI<sub>5</sub> are similar indicating that a close relationship between actinorubin and EI<sub>5</sub>. Further both substances are soluble in water and acid-methanol, insoluble in ether and acetone, resist boiling, and, as shown later, have similar toxicities for mice.

G. *The Antibacterial Activity of Antibiotic EI<sub>5</sub> for the Mycobacteria.* By use of the method described by Youmans<sup>12</sup> EI<sub>5</sub> was shown to be bacteriostatic for *M. tuberculosis*

H37Rv in a dilution of 1:100,000 and for *Mycobacterium phlei* in a dilution of 1:500,000.

H. *Toxicity of EI<sub>5</sub> for Mice.* Large doses of EI<sub>5</sub> ranging from 150,000 to 270,000 units per kg in the form of both the initial concentrate and purified antibiotic were injected intraperitoneally into 10 white mice weighing 20 to 35 g. One animal died on the 5th day and the others were sacrificed from the 5th

<sup>12</sup> Youmans G. P., *Proc. Soc. Exp. Biol. and Med.*, 1944, 57, 119.

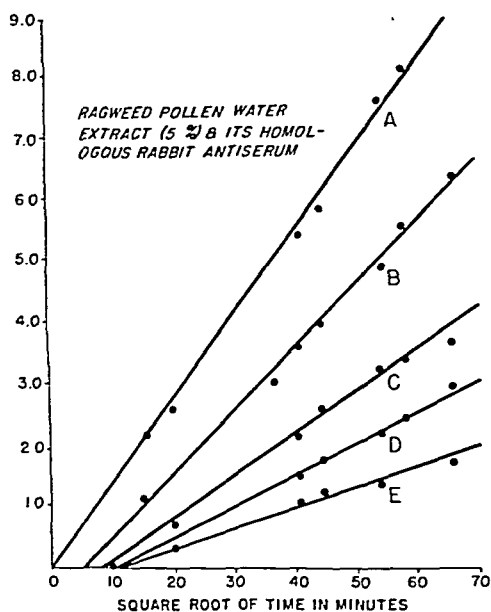


FIG. 1.

Movement of 5 bands of precipitate obtained with ragweed pollen extract and its homologous antiserum plotted against the square root of the time.

least 5 distinct substances in ragweed pollen extracts which are antigenic for rabbits. That the antigenic complexity may be even greater than this is indicated by the observation

that complete absorption of the ragweed extract with rabbit antiserum markedly reduces the direct skin reactivity in specifically sensitive human beings, but does not abolish it.<sup>13</sup> The relationship of these 5 antigens to the various chemical<sup>1</sup> and electrophoretic<sup>2</sup> fractions shown to exist in ragweed pollen extracts is not known at present, and more work is needed to elucidate this problem. The technic of Oudin shows great promise in the fractionation of antigenic mixtures. It has been applied by Oudin to the fractionation of horse serum<sup>6</sup> and by us to demonstrate the presence of at least 3 antigens in cytochrome c of 0.34% iron content.<sup>14</sup> Moreover, work from Oudin's laboratories<sup>9</sup> and from our own (unpublished experiments) indicates that it may be possible to apply his method as a quantitative tool for the determination of either antigen or antibody concentrations.

**Conclusion.** Application of the technic of Oudin to ragweed pollen extracts and their antisera demonstrated the presence of at least 5 different antigens in such extracts.

<sup>13</sup> Becker, E., and Marshall, R., unpublished work.

<sup>14</sup> Becker, E., and Munoz, J., *J. Immunol.*, 1949, **63**, 173.

Received June 15, 1949. P.S.E.B.M., 1949, **72**.

## Allergic Encephalitis Following Injection of Dialysate of Brain Tissue. (17410)

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(Introduced by K. Habel)

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The encephalitic process which follows the injection into monkeys<sup>1,2</sup> and guinea pigs<sup>3,4</sup> of homologous or heterologous brain tissue has been variously termed meningo-encephalomyelitis,<sup>7</sup> acute disseminated encephalomyelitis,<sup>1</sup> isoallergic encephalomyelitis<sup>3</sup> or allergic encephalitis,<sup>5</sup> and has been compared with the

post-vaccinal (rabies) paralysis of human beings.<sup>10</sup> The nature of the factor in brain

<sup>1</sup> Kabat, E. A., Wolf, A., and Bezer, A. E., *J. Exp. Med.*, 1947, **85**, 117.

<sup>2</sup> Morgan, I. M., *J. Exp. Med.*, 1947, **85**, 131.

<sup>3</sup> Freund, J., Stern, E. R., and Pisani, T. M., *J. Immunol.*, 1947, **57**, 179.

<sup>4</sup> Kopeloff, L. M., and Kopeloff, N., *J. Immunol.*, 1947, **57**, 229.

<sup>5</sup> Bell, J. F., Wright, J. T., and Habel, K., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 457.

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† Laboratory of Pathology.

‡ Died May 14, 1949. Laboratory of Biologics Control.

TABLE I.  
Supernatant Tests on Ragweed Pollen Extract and Its Homologous Rabbit Antiserum.

Tube No.	Ragweed extract, ml	Precipitable N in mg per ml of antiserum	Precipitin test on supernatant (ring method)	
			Antigen	Antibody
1	.04	.060	—	+
2	.08	.086	—	+
3	.12	.120	—	+
4	.16	.126	—	+
5	.20	.140	±	+
6	.30	.156	+	+
7	.40	.166	+	+

more than one band. The number of such bands corresponds to the minimum number of antigen and antibody systems present. Before applying this technic to ragweed pollen extracts the findings of Oudin were confirmed using crystalline antigens and antisera of known purity. This latter work will be published elsewhere.

*Materials and methods.* Defatted short ragweed (*Ambrosia elatior*) pollen was extracted with distilled water or physiological saline in the proportions of 5 g per 100 cc (5% extract) or 10 g per 100 cc (10% extract). The water or saline used in the extraction contained 1:5000 merthiolate (Lilly). The rabbits were immunized by repeated subcutaneous injections of the ragweed pollen extract using Freund's adjuvant technic.<sup>3,10</sup> Before use in the Oudin test the extracts were brought to pH 6.5-7.5 with 0.1N sodium hydroxide, and any precipitate which formed was filtered off. The procedure followed in the Oudin technic was essentially that described by him<sup>7</sup> with only minor modifications. One part of 0.6% Bacto agar (Difco) in physiological saline containing 1:5000 merthiolate was used to mix with one part of antiserum. The agar was kept at 48-50°C and the serum was brought to that temperature before mixing thoroughly with the agar. Precipitin tubes of 3 mm inside diameter and approximately 5-6 cm long, which had been previously coated with 1% agar and dried under vacuum, were filled 1/2 to 2/3 full with the serum-agar mixture. The tubes containing the serum-agar were then placed in the refrigerator for

5 to 10 minutes to allow the agar to solidify. The antigen solution containing 1:10,000 merthiolate was now layered on top of the solid serum-agar mixture, and the tubes were sealed with plasticine to prevent evaporation and kept for 2 weeks at approximately 25°C.

*Results.* Quantitative precipitin tests were set up using rabbit antiserum and short ragweed pollen extract in 50% glycerin in the same manner as reported by Bukantz *et al.*<sup>11</sup> The results of the supernatant analysis shown in Table I demonstrate that more than one antigen-antibody system exists, since both antigen and antibody were found in the same supernatant. (See Kendall.<sup>12</sup>) The Oudin test was run using a 10% extract of ragweed pollen in physiological saline. The final concentration of antiserum in the serum-agar mixtures was 50%. An antiserum, in a 50% final concentration, when tested with 5% water extract of the same batch of pollen gave 5 bands. The position of each individual band was measured at 6-18 hour intervals with calipers accurate to 0.1 mm. The results of plotting the distance moved by each of the bands against the square root of the time gave, as found by Oudin with other systems, straight lines (Fig. 1). These experiments were repeated using different antisera with similar results. In some experiments there was an indication of the presence of a sixth band, but this was so faint that one could not be certain of its actual existence.

*Discussion.* The results of these experiments clearly demonstrate that there are at

<sup>10</sup> Freund, J., and Bonanto, M., *J. Immunol.* 1944, **48**, 325.

<sup>11</sup> Bukantz, S., Johnson, M., and Hampton, S., *J. Allergy*, 1949, **20**, 1.

<sup>12</sup> Kendall, F. E., *J. Clin. Invest.*, 1937, **16**, 921.

TABLE I.  
Allergic Encephalitis Produced in Guinea Pigs by Various Fractions of Brain Tissue.

Exp. No.	Material*	Amt inj.,† mg	Diagnosis‡		
			Clinical		Histopathological
			Positive	Doubtful	Positive
216-A	Cal acetate extr. of normal rabbit brain before dialysis	50	2/6	2/6	1/6
B	Dialysate of normal rabbit brain	50	9/20	8/20	12/18
254	Dialysate of normal rabbit brain	40	3/5	0/5	3/3
241	(Same)	25	2/10	2/10	2/2
233-A	Water extr. of normal beef brain before dialysis	100	6/9	2/9	1/3
251-B	Dialysate of normal beef brain	250	2/10	2/10	5/10
233-B	(Same)	100	2/9	2/9	1/3
D	(Same)	50	1/5	0/5	1/1

\* All tissues were previously dried and extracted with benzene and ether.

† Expressed as equivalent weight of wet whole brain.

‡ Denominator of fraction indicates number of animals in each experiment.

litis. In no instance, however, did all of the animals injected with any one preparation develop the characteristic signs or lesions of the disease. Since the incidence of this disease following one dose of whole brain in adjuvant has been variable<sup>4</sup> no attempt was made to determine the amount of factor present by the incidence of positive reactions in animals. All observations which showed a positive picture of allergic encephalitis are regarded as significant.

On the basis of the above it is evident that the substance which causes allergic encephalitis is capable of passing through a cellophane membrane and, therefore, has a molecular weight of approximately 10,000 or less. It will be recalled that Alvord<sup>7</sup> found the factor to be a heat-stable, phosphatide-like substance

which might possibly resemble the alcohol-soluble brain-hapten of Lewis<sup>8</sup> and Rudy.<sup>9</sup> Very little is known regarding the properties of the materials recovered in the dialysate, except that the mixture contains salts, amino acids and other substances and is Biuret negative at a concentration equivalent to 200% brain tissue, *i.e.*, a solution with 4 to 8% total solids.

*Summary.* The dialysate of benzene-ether extracted brain tissue is capable of causing signs and lesions of allergic encephalitis in guinea pigs. The material recovered in the dialysate is free of protein and presumably contains only substances of low molecular weight.

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which is responsible for this process is not known, although reports have appeared showing that the factor is present in the phosphatide fraction of brain<sup>7,11</sup> and in the protein fraction remaining after extraction of all brain lipoids.<sup>11</sup> Work on the purification of rabies vaccine<sup>5</sup> has shown that the factor which causes allergic encephalitis is soluble in a solution of calcium acetate. This factor was separated from rabies vaccine by washing the infected brain tissue carefully with calcium acetate solution after extraction with benzene and ether. When incorporated into the mineral oil adjuvant described by Freund and McDermott,<sup>6</sup> the separated factor produced allergic encephalitis in guinea pigs. This report describes some observations made during a study of the properties of the factor causing allergic encephalitis.

The procedure by which dialysates of brain tissue were prepared is an extension of the method<sup>5</sup> for freeing rabies vaccine from the factor causing allergic encephalitis. Brain tissues from rabbits and cattle were minced in a Waring Blendor, suspended in distilled water, and the suspensions dried from the frozen state. The dried brain was extracted with benzene followed by two extractions with ether. After removal of the ether, the brain tissue was suspended in distilled water. When the suspension had become thoroughly hydrated, it was further diluted with an equal volume of M/5 calcium acetate solution, or in some experiments with distilled water. The mixture was placed in cellophane tubing and suspended in 4 volumes of distilled water. All cellophane tubes were tested by filling them with water and observing for leaks while moderate pressure was applied to the top of the tubes. Care was taken to wash the end of the tubing through which brain suspension was introduced so that the dialysate would not

be contaminated. After storage at 6° C for 3 to 4 days with frequent agitation, the clear dialysate, which now had a slightly amber tint, was removed and dried from the frozen state. This dialysate usually contained 2 to 4 mg of solids per milliliter at a concentration equivalent to an original 10% brain emulsion.

The presence of the allergic factor in the brain dialysate was determined by mixing a 5 ml sample with 5 ml of the adjuvant (4.5 ml of mineral oil containing 9 mg of dried acid fast bacilli [B.C.G.], plus 0.5 ml Arlacel A) and injecting 1 ml of the mixture subcutaneously into each 200-250 g guinea pig. The animals were observed for at least a month following injection. Those which developed only slight weakness in the hind quarters are recorded as having doubtful symptoms, those with definite weakness or paralysis as positive. Forty-six of the 74 animals used were killed for histological study in various stages of the disease, after clinical recovery, or after 30 to 60 days had elapsed without clinical evidence of allergic encephalitis. Therefore, final mortality figures could not be tabulated. Brains and spinal cords were fixed in formalin and sections through the thalamus, pons, and several levels of the cord were examined. The lesions found were similar in all respects to those previously produced by the injection of unmodified brain tissue in adjuvant. They consisted of scattered, perivascular cuffs of mononuclear and microglial cells about venules in the cerebral white matter, hippocampus, thalamus, pons, cerebellar white matter, and cord. The size and number of the lesions varied greatly in individual animals.

Brain preparations which produced either positive clinical signs or characteristic microscopic brain lesions were listed as containing the factor. In most cases a positive histopathological diagnosis was related to positive clinical signs. In a few cases characteristic lesions were found in animals which showed no clinical signs of the disease. The results of 6 experiments on dialysis are summarized in the accompanying table.

It will be noted that animals injected with the brain preparations either before or after dialysis showed evidence of allergic encephal-

<sup>6</sup> Freund, J., and McDermott, K., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 548.

<sup>7</sup> Alford, E. C., *J. Immunol.*, 1949, **61**, 355.

<sup>8</sup> Lewis, J. H., *J. Immunol.*, 1934, **26**, 331.

<sup>9</sup> Rudy, H., *Biochem. Z.*, 1933, **267**, 77.

<sup>10</sup> Stuart, G., and Krikorian, K. S., *Ann. Trop. Med. Parasit.*, 1928, **22**, 327.

<sup>11</sup> Koprowski, H., and Jervis, G. A., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 472.

TABLE I.

Effect of Rose Bengal and U.V. Light Alone and in Combination on Urinary Coproporphyrin Excretion in Rabbits ( $\gamma$  per 24 hr).

Day	R1	R2	R3	R4	R5	Control 1	Control 2
0	7.0	6.2	6.4	9.7	5.8	7.8	6.6
	Rose Bengal and U.V. light						
1	300.0	110.0	120.0	150.0	135.0	8.6	5.2
2	80.0	96.6	53.2	55.0	76.8	14.0	6.3
3	70.0	70.0	25.0	38.6	62.8	9.0	7.2
4	30.0	12.0	15.0	38.7	15.7	6.8	7.4
5	7.2	10.0	10.0	7.7	13.8	9.4	6.8
6	9.4	5.0	—	15.3	9.7	8.6	8.1
7	10.0	6.3	15.6	21.3	9.8	6.5	7.3
8	10.4	7.8	12.0	16.7	10.1	5.8	6.5
9	10.1	9.4	11.0	16.5	8.7	7.6	7.3

TABLE II.

Effect of Hemolysis Produced by Distilled Water or Phenylhydrazine Hydrochloride on Urinary Coproporphyrin Excretion in Rabbits.

Day	Urinary Coproporphyrin in $\gamma$ in 24 hr		
	R6	R7	R8
0	7.3	9.7	10.0
	Distilled water	Phenylhydrazine alone	Phenylhydrazine with U.V. light
1	4.1	10.0	9.9
2	9.7	12.0	10.0
3	11.0	16.0	4.5
4	10.8	27.0	10.9
5	9.7	28.0	28.0
6	8.4	26.1	42.0
7		40.7	24.0

quantity to cause a rapid and intense hemolysis in one rabbit. As seen in Table II, there was little if any effect on the coproporphyrin excretion.

For the same purpose 30 mg of phenylhydrazine hydrochloride per kg were injected subcutaneously into 2 rabbits. One of these animals (R8) was exposed to U.V. light for 25 minutes immediately after phenylhydrazine administration.

The mild delayed increase noted in the phenylhydrazine experiments in Table II is probably related to increased erythropoietic activity, as previously found by Schwartz and co-workers.<sup>6</sup>

**Summary and conclusions.** The administration of Rose Bengal and ultraviolet irradiation in rabbits results in rapid and marked increase in the excretion of urinary coproporphyrin

Type III to values 17 to 40 times those in the controls, returning to normal by the 5th day. Neither the dye alone nor the irradiation alone had any effect.

Simple hemolysis by distilled water had no effect upon the urinary coproporphyrin excretion. Phenylhydrazine produced a slight delayed increase of coproporphyrin excretion believed to be due largely to an increase of coproporphyrin Type I, as a result of increased erythropoiesis.

Additional studies are being undertaken in an attempt to discover the origin of the coproporphyrin excreted and the effects of other photodynamic compounds.

These results constitute the first demonstration that photodynamic activity causes a marked increase in the excretion of coproporphyrin III.

<sup>6</sup> Schwartz, S., Glickman, M., Hunter, R., and Wallace, J., AEC-D-2109.

## Effect of Rose Bengal and Ultra Violet Light on Porphyrin Excretion in Rabbits.\* (17411)

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The photodynamic effect of porphyrins *in vitro* and *in vivo* is well known,<sup>1</sup> as is the relationship of sunlight to so called photosensitive or congenital porphyria. In 10 cases of *Pemphigus foliaceus* characterized by photosensitivity, the author has also found large increases in excretion of urinary coproporphyrin.† The question arose as to whether a heightened urinary porphyrin excretion might be the effect of photodynamic activity, rather than, as always hitherto supposed, that the excessive porphyrin was the cause of this activity. The well known occurrence of marked increases of urinary coproporphyrin III in a variety of disease states,<sup>2</sup> without photosensitivity, in fact suggested that photodynamic activity in the *Pemphigus foliaceus* cases might be causing the coproporphyrinuria.

Rose Bengal is also known to exert a powerful photodynamic action.<sup>3</sup> In studying the fundamental relationship of light to porphyrin excretion the effect of previous sensitization by Rose Bengal and other photosensitizing compounds was investigated. Some of the results are the basis for the present report.

**Methods.** Adult rabbits of either sex were injected intravenously with a dose of 100 mg of Rose Bengal per kilo of body weight. The dye was dissolved in 10 cc of physiological saline solution.

Unfiltered light from a CH-4 Mercury arc lamp§ of maximum near ultraviolet intensity

at 3650 Å, was used for the photosensitization. This wave length corresponds with the maximum absorption of the dye. The hair was shaved from the animal's back and the shaved area was irradiated immediately following the administration of Rose Bengal for 25 minutes at a distance of 12 inches, the approximate focal distance of the lamp.

The concentration of total urinary coproporphyrin was determined with a Lumetron fluorophotometer, in other respects following the technic of Schwartz, Hawkinson, Cohen, and Watson.<sup>4</sup> Their method of isomer analysis<sup>4</sup> was also employed. Crystallization of coproporphyrin methyl ester was done according to methods used previously in this laboratory.<sup>5</sup>

**Results.** The results are given in Table I. The zero value in each instance serves as a control. Rabbits R1-R5 received both Rose Bengal and ultraviolet light at the beginning of day number 1. Control Rabbit No. 1 was injected with Rose Bengal in the same concentration, but the animal was kept in darkness. Control Rabbit No. 2 received ultraviolet irradiation but no Rose Bengal.

Isomer analysis of the coproporphyrin from Rabbit 1 for the first 24 hours after the photosensitization, revealed 96% coproporphyrin type III, indicating that most of the increase was of this isomer. The methyl ester crystallized in typical rosettes melting at 145°, thus confirming the isomer analysis.

The fecal coproporphyrin was determined during the first 4-day periods in 3 rabbits. No increased values were found.

To control the factor of hemolysis, distilled water was injected intravenously in sufficient

\* Aided by Research Grant No. 345, U. S. Public Health Service.

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<sup>1</sup> Blum, H. F., Photodynamic actions and disease caused by light, Reinhold Pub. Corp., 1941.

‡ Unpublished.

<sup>2</sup> Watson, C. J., and Larson, E., *Physiol. Rev.*, 1947, **27**, 478.

<sup>3</sup> Fieissinger, N., Albeaux Fernet, M., Aussanaire, M., *Bull. Soc. Franc. Derm. et Syph.*, 1937, **44**, 1768.

§ Black Light Products, Inc., Chicago, Ill., Model No. B1-2.

<sup>4</sup> Schwartz, S., Hawkinson, V., Cohen, S., and Watson, C. J., *J. Biol. Chem.*, 1947, **168**, 133.

<sup>5</sup> Grinstein, M., Schwartz, S., and Watson, C. J., *J. Biol. Chem.*, 1945, **157**, 323.

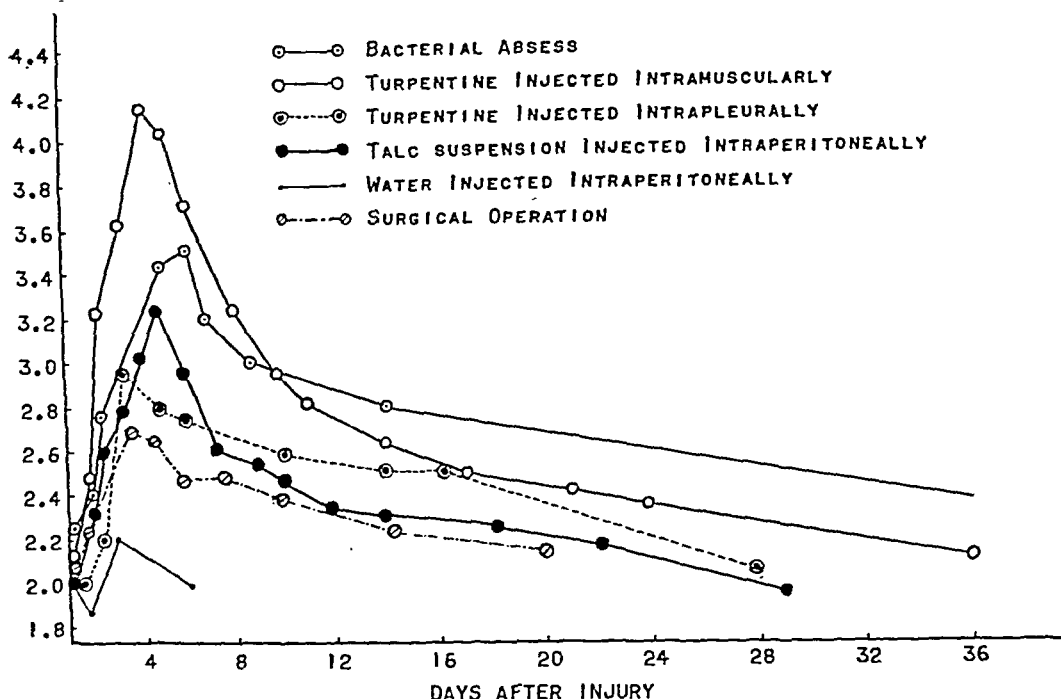


FIG. 1. NON-GLUCOSAMINE SERUM POLYSACCHARIDE OF DOGS EXPRESSED AS A PERCENTAGE OF SERUM PROTEIN FOLLOWING INJURY.

claving for 30 minutes at 15 lb pressure. The turpentine was then transferred to a syringe and injected into the experimental animals, using aseptic technic. In one series of studies the turpentine was injected into the pleural cavity, and in a second series it was injected intramuscularly. Opie<sup>5</sup> describes the former type of injection as "resulting in serofibrinous inflammation which undergoes resolution, so that the pleural cavity is restored to normal in about 10 days; there is no destruction of tissue."

Intramuscular injection of turpentine on the other hand results in a sterile abscess which is filled with purulent material. In this case there is much destruction of tissue early in the course and repair by granulation and fibrosis after evacuation or absorption of the contents of the abscess. In our experiments, using mongrel dogs, swelling usually became apparent after 24 hours, reached a maximum at 48-72 hours, and was reduced after 5 days. The temperature was usually elevated after

24 hours, reached a maximum within 48 hours, and became normal by the fifth day. Abscess formation was noted in every case; in approximately one-half of the experiments the abscess ruptured spontaneously and drained about the fourth day. In these cases it was apparent that considerable tissue damage resulted; the formation of some repair tissue had taken place by the fourth day, when rupture occurred. Average results of the polysaccharide expressed as percentage of protein for 6 animals are expressed graphically in Fig. 1. In both types of injection the serum polysaccharide levels reached maximal elevation on the fourth day, however, the animals injected intramuscularly exhibited the higher maxima.

**Talc Granuloma.** A talc suspension for injection was prepared by suspending 20 g of talc in 80 ml of water. The suspension was sterilized, allowed to cool, resuspended and injected intraperitoneally into healthy mongrel dogs through a No. 15 needle, using aseptic technic. The signs of peritoneal ir-

<sup>5</sup> Opie, E. L., *Arch. Int. Med.*, 1910, 541.



## Serum Polysaccharide Levels in Experimental Inflammation.\* (17412)

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The serum polysaccharide level has been shown to be elevated in malignancies, tuberculosis, and certain other pathological conditions. It has been suggested by Seibert, Seibert, Atno, and Campbell<sup>1</sup> that this rise is related to tissue destruction. They note that the  $\alpha_2$  globulin, which is especially rich in carbohydrate content, is elevated in tuberculosis and carcinomas. Since Shedlovsky and Scudder<sup>2</sup> have noted that a rise in  $\alpha_2$  globulin is associated with inflammation or tissue destruction irrespective of cause, one might postulate that a rise in serum polysaccharides is to be expected wherever inflammation occurs. In studies of human conditions this has, in general, proved to be true,<sup>3</sup> although low results were found in some infectious diseases. It appeared that this problem might be further elucidated by a study of inflammation in experimental animals.

**Experimental. Chemical methods.** In this study non-glucosamine polysaccharide and protein were determined as previously described.<sup>4</sup> Glucosamine polysaccharide was also determined on all samples, but as the results roughly parallel those for non-glucosamine polysaccharide, they are not given in this paper.

**Inflammation due to infection.** In order to

\* This work was supported by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council and by funds from the John Areher Hatchett Memorial Fund.

<sup>1</sup> Seibert, F. B., Seibert, M. V., Atno, A. J., and Campbell, H. W., *J. Clin. Invest.*, 1947, **26**, 90.

<sup>2</sup> Shedlovsky, T., and Scudder, J., *J. Exp. Med.*, 1942, **75**, 119.

<sup>3</sup> Shetlar, M. R., Foster, J. V., Kelly, K. H., Shetlar, C. L., Bryan, R. S., and Everett, M. R., accepted for publication in *Cancer Research*.

<sup>4</sup> Shetlar, M. R., Foster, J. V., Kelly, K. H., and Everett, M. R., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 507.

produce an infectious lesion which could be closely followed, 5 ml of a mixed culture of *Staphylococcus aureus* and *Streptococcus pyogenes* and mucin† were injected subcutaneously into the back legs of healthy mongrel canines. In several cases, large abscesses about 10 cm in diameter resulted. The abscesses usually ruptured spontaneously and drained on about the sixth day; the skin sloughed on the eighth day, leaving the abscess area lined with granulation tissue. The wounds granulated completely within 60 days. The temperature reached a maximal elevation on the third day, but did not return to normal until the sixth day. Clinically, there was no sign of septicemia, and the animals appeared greatly improved by the sixth day. The non-glucosamine polysaccharide level was elevated slightly on the first day, when expressed as a percentage of protein, but did not reach a maximum until the sixth day. The level then fell slowly, but it was still elevated appreciably on the 26th day and did not become normal until the 52nd day. Results obtained from the study of 3 dogs are depicted graphically in Fig. 1. Similar results were not obtained with all dogs, as some animals exhibited more resistance to the infectious agent and no apparent abscess was formed. In these cases the maximal polysaccharide level was not so high, and the return to normal occurred much earlier.

**Turpentine Inflammation.** Turpentine was sterilized for injection by filtering National Formulary grade turpentine through a Seitz filter by suction into a filter flask. The flask and filter were previously sterilized by auto-

† The authors wish to gratefully acknowledge the aid of Dr. Florene Kelly and Dr. Homer Marsh, Department of Bacteriology, Oklahoma University School of Medicine, for their advice in planning the bacterial phase of this work and for their preparation of the culture used.

## Hydrolysis of Conjugated Steroids by Bacterial Glucuronidase. (17413)

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The use of acid for liberating the steroids of urine is based on the observation that these compounds are excreted in conjugated form as evidenced by their insolubility in fat solvents and by the isolation of steroid glucuronides<sup>1-3</sup> and sulfates.<sup>4-6</sup> The evidence that such treatment causes considerable loss<sup>7-10</sup> has led to a consideration of enzymes for the liberation of steroids from their conjugates.

Cohen and Marrian<sup>11</sup> and Patterson<sup>12</sup> have previously reported the liberation of estrogen of pregnancy urine by bacterial action and Barker, Brooksbank, and Haslewood<sup>13</sup> have found that pregnanediol glucuronide, and in some experiments glucuronic acid, which presumably was liberated from the conjugate were destroyed by some strains of *Staphylococcus albus*. Preparations possessing glucuronidase activity which were obtained from mammalian tissues hydrolyze the glucuronides of estriol,<sup>14,15</sup> pregnanediol<sup>7,16</sup> and other compounds<sup>14,17,18</sup> and Cohen and Bates<sup>19</sup> have

studied the hydrolysis of the sulfuric acid esters of estrogens by an enzyme, phenolsulfatase, obtained from a mold, *Aspergillus oryzae*.\*

Recently, we have reported<sup>20</sup> the production of glucuronidase by *E. coli* cultured in the presence of menthol glucuronide and an adequate oxygen supply. By this means potent culture filtrates were obtained which may be used without further purification or concentration. The present report deals primarily with the effect of this bacterial glucuronidase on the hydrolysis of some of the urinary steroids.

**Experimental Procedures.** The urines, preserved with thymol, were boiled at neutral pH for 10 minutes. Single specimens were boiled immediately after voiding and 24-hour samples at the end of the collection period during which time they were kept at 5°C. This precaution was taken because of the reports that glucuronidase<sup>21</sup> and phenolsulfatase<sup>22</sup> occur in urine. Free steroids were determined in these urines and the amounts of

<sup>1</sup> Cohen, S. L., and Marrian, G. F., *J. Soc. Chem. Ind.*, 1935, **54**, 1025.

<sup>2</sup> Venning, E. H., and Browne, J. S. L., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 792.

<sup>3</sup> Mason, H. L., and Strickler, H. S., *J. Biol. Chem.*, 1947, **171**, 543.

<sup>4</sup> Schachter, B., and Marrian, G. F., *J. Biol. Chem.*, 1938, **126**, 663.

<sup>5</sup> Munson, P. L., Gallagher, T. F., and Koch, F. C., *Endocrinology*, 1942, **30**, S1036.

<sup>6</sup> Venning, E. H., Hoffman, M. M., and Browne, J. S. L., *J. Biol. Chem.*, 1942, **146**, 369.

<sup>7</sup> Talbot, N. B., Ryan, J., and Wolfe, J. K., *J. Biol. Chem.*, 1943, **148**, 593.

<sup>8</sup> Astwood, E. B., and Jones, G. E. S., *J. Biol. Chem.*, 1941, **137**, 397.

<sup>9</sup> Stimmel, B. F., *J. Biol. Chem.*, 1949, **178**, 217.

<sup>10</sup> Bitman, J., and Cohen, S. L., *J. Biol. Chem.*, 1949, **179**, 455.

<sup>11</sup> Cohen, S. L., and Marrian, G. F., *Biochem. J.*, 1934, **28**, 1603.

<sup>12</sup> Patterson, J., *Brit. Med. J.*, 1937, **2**, 522.

<sup>13</sup> Barker, M., Brooksbank, B. W. L., and Haslewood, G. A. D., *Nature*, 1948, **162**, 701.

<sup>14</sup> Fishman, W. H., *J. Biol. Chem.*, 1939, **131**, 225.

<sup>15</sup> Grant, J. K., and Marrian, G. F., *Biochem. J.*, 1948, **43**, V.

<sup>16</sup> Mason, H. L., and Kepler, E. J., *J. Biol. Chem.*, 1945, **161**, 235.

<sup>17</sup> Talalay, P., Fishman, W. H., and Huggins, C., *J. Biol. Chem.*, 1946, **166**, 757.

<sup>18</sup> Levy, G. A., *Nature*, 1947, **160**, 54.

<sup>19</sup> Cohen, H., and Bates, R. W., *Endocrinology*, 1949, **44**, 317. Program of the Association for the Study of Internal Secretions in Atlantic City on June 4, 1949.

\* Studies on hydrolysis of combined estrogens by phenolsulfatase which were discussed at the Federation meeting<sup>20</sup> in Detroit, April 1949. will be presented in detail in a subsequent report.

<sup>20</sup> Buchler, H. J., Katzman, P. A., and Doisy, E. A., *Fed. Proc.*, 1949, **8**, 189.

<sup>21</sup> Odell, L. D., Fishman, W. H., and Hepner, W. R., *Science*, 1948, **108**, 355.

<sup>22</sup> Huggins, C., and Smith, D. R., *J. Biol. Chem.*, 1947, **170**, 391.

ritation usually subsided in about 3 hours. No appreciable temperature elevation occurred during the time the animals were studied. Extensive granulomatous lesions resulted in all cases but no attempt was made to correlate the extent with serum polysaccharide levels. Average results of studies using 4 dogs are depicted in Fig. 1. The serum polysaccharide reached a maximal elevation on the third or fourth day and was higher than normal even on the 20th day. Intraperitoneal injection of 100 ml of sterile water in a control experiment caused only a slight change in the serum polysaccharide as noted in Fig. 1.

*Experimental Surgical Operation.* Several experimental laparotomies were carried out under aseptic conditions on mongrel dogs, and the serum polysaccharide level was studied by serial determinations following operation. Since no tissue was removed during these procedures, the only tissue injury was that resulting from the incisions. Data obtained from these studies based on the results from 3 animals are presented graphically in Fig. 1. The maximal elevation of serum polysaccharide occurred on the fourth day. The elevation was not as great as that caused by severe bacterial infection or turpentine abscess. No significant temperature elevation was found.

*Discussion.* It is apparent from the data presented that an increase in serum polysaccharide follows the production of experimental inflammation regardless of the causative agent. As this response is a delayed one, with maximal elevation in 3 to 6 days after injury, the polysaccharide level does not appear to be directly correlated with tissue destruction. Furthermore, the same effect is produced by

inflammatory processes caused by injection of talc suspensions or by intrapleural injection of turpentine, conditions in which little tissue destruction is thought to occur. Fever is apparently not essential for the elevation, inasmuch as the serum polysaccharide rose in cases where no temperature elevation occurred. It is therefore possible that the elevation of polysaccharide may be correlated with tissue proliferation or repair.

The length of time which elapsed before the polysaccharide level became normal again seemed to have some connection with the extent of repair. The time required for the polysaccharide level to become normal in an animal with a large abscess resulting from bacterial action was much longer than for types of inflammation where less regeneration of tissue occurred. It seems unlikely that the continued high level could be the direct result of tissue injury, as signs of infection and trauma were absent after the eighth day.

*Summary and conclusions.* Studies were made of serum polysaccharide levels in dogs following the production of sterile turpentine abscesses, of bacterial abscesses, and of talc granulomas, the injection of turpentine intrapleurally, and experimental surgical operations. In all cases the polysaccharide level rose and the maximal level depended somewhat upon the type of inflammation. Elevation occurred both in the presence and absence of fever. The maximal elevation appeared within 3 to 6 days after the initial injury. The suggestion is made that this phenomenon is correlated in some way with tissue proliferation or repair.

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TABLE II.  
Comparison of Hydrochloric Acid and Glucuronidase Hydrolysis of the Ketosteroids of Human Urine.

Urine No.	Method of hydrolysis		
	HCl	Glucuronidase	No hydrolysis
Normal men—mg* per 24 hr			
1	—	4.25	.488†
2	—	8.3	1.176†
3	—	7.2	.612†
4	—	4.05	.450†
5	—	4.75	.546†
6	14.4	6.7	.60†
7	11.0	5.1	.416†
8‡	26.2	14.0	.448†
Pregnant women—mg* per l			
1§	0.92	1.2	.07
2	5.5	5.0	.18
3	5.8	5.0	.26
4	10.8	7.4	.42
5	6.3	5.6	.21
6	4.8	3.6	.16

\* Expressed as dehydroisoandrosterone.

† Micro-Girard separation not made.

‡ Pseudohermaphrodite.

§ Pregnancy urines 1-6 are the same as those recorded in Table I.

ketosteroid conjugated with sulfuric acid accounts for at least a part of this difference but here again, it is surprising to find so large a proportion of the ketosteroid fraction in combination with glucuronic acid.

The quantities of free estrogens and ketosteroids recorded in Tables I and II are of interest since they were obtained from fresh urine in which enzymes, if present, had been destroyed by boiling. However, even these low values may be due to enzymic hydrolysis within the bladder.

As would be expected, bacterial glucuronidase hydrolyzed sodium pregnanediol glucuronide resulting in a 90% recovery of pregnanediol as determined by the method described by Talbot, *et al.*<sup>29</sup>

Dr. Ralph A. Kinsella, Jr. of this department, in preliminary studies has found that

glucuronidase markedly increases the amount of cortical steroids of the urine of normal adult men as determined by a somewhat modified method of Heard, *et al.*<sup>30</sup>

**Summary.** Bacterial glucuronidase which hydrolyzes the glucuronides of estriol, pregnanediol, menthol and phenolphthalein liberates a large proportion of estrogen, ketosteroid and corticosteroid of human urine.

We are deeply indebted to Professor G. F. Marrian for estriol glucuronide; to Dr. A. Stanley Cook of Ayerst, McKenna and Harrison for estrone sulfate; to Dr. H. L. Mason for dehydroisoandrosterone sulfate; to Dr. Willard Allen for pregnanediol glucuronide, and to Miss Corinne Dewes, Robert A. Doisy, and Joseph Yglesias for invaluable laboratory assistance.

<sup>30</sup> Heard, R. D. H., Sobel, H., and Venning, E. H., *J. Biol. Chem.*, 1946, **165**, 699.

<sup>29</sup> Talbot, N. B., Berman, R. A., MacLachlan, E. A., and Wolfe, J. K., *J. Clin. Endocrinology*, 1941, **1**, 668.

Received August 1, 1949. P.S.E.B.M., 1949, **72**.

TABLE I.  
Comparison of the Influence of Different Hydrolytic Agents on Total Estrogen Liberated from Human Pregnancy Urine.\*

Method of hydrolysis				Total estrogen as mouse units of estriol per l		
Urine	Total estrogen (Kober) as mg estrone per l			Urine	Total estrogen as mouse units of estriol per l	
	HCl	Glucuronidase	None		HCl ×1000	Glucuronidase ×1000
1	5.38	4.88	.03	A†	109	147
2	16.27	15.00	.21	B†	155	207
3	10.5	12.3	.24	C†	120	213
4	13.87	17.25	.285	D†	320	460
5	6.75	9.75	.15	E†	176	266
6	12.0	13.3	.06			

\* Obtained during the last month of pregnancy.

† Ether extracts of urines A-E were not fractionated into the phenolic and neutral fractions before assay. In the bioassay (Marrian), 20 animals per dose were used and estriol was used for the standard.<sup>2</sup>

estrogen and 17-ketosteroid liberated from their conjugates with glucuronidase compared with the amounts liberated by acid hydrolysis.

Acid hydrolysis was performed by refluxing the urine for 10 minutes with 15 vol. % concentrated HCl. For enzymic hydrolysis, the urines with added glucuronidase were incubated for 24 hours at 38°C at pH 6.5. All hydrolyzed and unhydrolyzed samples were adjusted to pH 3 and immediately extracted with ether. Phenolic and neutral fractions were separated according to Friedgood, *et al.*,<sup>23</sup> ketosteroids determined by the method of Holtorf and Koch<sup>24</sup> after removing the non-ketonic fraction<sup>25</sup> and total estrogens by Salter's<sup>26</sup> modification of the Kober method.

The extracts obtained from the glucuronidase hydrolyzed urine gave very much less background color with the Kober reagent (absorption at 420 mμ) and a more typical color with Zimmermann's reagent than that obtained after acid hydrolysis. Furthermore, a larger proportion of the color with m-dinitrobenzene is due to ketonic compounds reacting with Girard reagent T.

Under our experimental conditions, glu-

curonidase quantitatively splits glucuronic acid from menthol glucuronide as measured by Fishman's modification<sup>27</sup> of Miller and Van Slyke's method,<sup>28</sup> and hydrolyzes 95% of estriol glucuronide. Furthermore, glucuronidase preparations tested with estrone sulfate and dehydroisoandrosterone sulfate contained neither phenolic nor alcoholic sulfatase.

**Results.** The data in Table I show that except for urines 1 and 2, higher values for total estrogen were obtained after hydrolysis with glucuronidase than with hydrochloric acid. In addition, the estimation is more accurate because of the lower background color after treatment with Kober's reagent. Although the lower values after acid hydrolysis may be due to incomplete hydrolysis as well as destruction, it is surprising that so large an amount of estrogen is conjugated with glucuronic acid.

With the urine of pregnant women as well as that of normal men and one case of pseudohermaphroditism, glucuronidase did not liberate as much ketosteroid as that liberated by hydrochloric acid (Table II). Undoubtedly

<sup>23</sup> Friedgood, H. B., Garst, J. B., and Haagen-Smit, A. J., *J. Biol. Chem.*, 1948, **174**, 523.

<sup>24</sup> Holtorf, A. F., and Koch, F. C., *J. Biol. Chem.*, 1940, **135**, 377.

<sup>25</sup> Pineus, G., and Pearlman, W. H., *Endocrinology*, 1941, **29**, 413.

<sup>26</sup> Salter, W. T., Humm, F. D., and Oesterling, M. J., *J. Clin. Endocrinology*, 1948, **8**, 295.

<sup>27</sup> Fishman, W. H., *J. Biol. Chem.*, 1939, **127**, 367.

<sup>28</sup> Miller, B. F., and Van Slyke, D. D., *J. Biol. Chem.*, 1936, **114**, 583.

TABLE I.

Procedures									
Group	No. of rats	Kind of inj.	Autopsy body wt, g	Ovaries			Uteri		
				Wt in mg	S.R.	Odds	Wt in mg	S.R.	Odds
1	20	G.	63	Immature females					
	20	G. & P.	57	139	2.0	2-1	136	2.0	20-1
2	10	S.	81	23	1.2	7-1	68	2	10-1
	10	P.	81	19			55		
3	21	St.	103	Immature females: ovariectomized					
	21	St. & P.	102	—			250	3.5	120-1
4	16	S.	147	Mature females					
	16	P.	140	51	1.0	4-1	186	3.0	60-1
5	19	S.	206	70	2.7	100-1	410	1.5	4-1
	19	P.	198	81			449		

The wet wt of the ovaries and uteri are shown. G = Gonadogen, P = Prostigmin, S = Saline controls, and St = Stilbesterol.

S.R.—Significant ratio which is based on S.D. (stand. dev.) =  $\frac{\sqrt{\sum d^2 f}}{n}$ ; S.E.—(standard error) =  $\frac{\text{S.D.}}{\sqrt{n}}$  and standard error of the difference =  $\sqrt{(S E_1)^2 + (S E_2)^2}$ .

for the uteri.

Some investigators<sup>11,12</sup> believe that in cases of paired data the method of Student<sup>11</sup> should be used. This method uses the standard deviation and by tables<sup>11,12</sup> the odds that such results will occur again can be determined. We have analyzed the data by this method as shown in Table I. It will be seen that the odds for Group 1 are 2-1 for the ovaries and 20-1 for the uteri. In contrast to this, Group 2 shows that prostigmin alone did not enlarge the uterus of the immature rat, in fact the mean weights of ovaries and uteri were somewhat smaller after prostigmin as compared with saline (0.9%) controls (Group 2 Table I). In other words the uterus must be in a congested state before the effects of prostigmin can be detected.

Group 3 consisted of ovariectomized virgin female rats in which the controls received 0.25 mg stilbesterol daily while the test animals

received 0.25 mg of stilbesterol plus 0.05 mg prostigmin daily. Treatment was begun 4 or 5 days after operation and was continued for 7 days. The animals were then similarly sacrificed and the uteri weighed and disiccated. This group was designed to test prostigmin on the hyperemic uterus in the absence of ovaries. It will be noted that the increase in weight of these uteri was at least as much as that found in Group 1 where the ovaries were present. The figures of 250 and 272 for the weights on the uteri give a difference of 8.8%. The "significant ratio" of 3.5 shows that this weight difference is significant and when analyzed by Student's method the odds are 120 to 1, which are the largest in Table I.

These experiments with immature females lead us to consideration of prostigmin studies in sexually mature female rats. These data are shown in Groups 4 and 5 (Table I). The only difference between these two groups is the duration of injection which was 7 and 30 days in Groups 4 and 5 respectively. Both

<sup>11</sup> Student, *Biometrika*, 1908, 6, 1.

<sup>12</sup> Love, H. H., *J. A. Soc. Agronomy*, 1924, 16, 70.

# Prostigmin on the Ovaries and Uteri of Albino Rats.\* (17414)

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Prostigmin is a parasympathomimetic drug that exerts some of its effects through its ability to inhibit cholinesterase and enhance the action of acetylcholine. Soskin, Wachtel, and Hechter<sup>1</sup> showed that prostigmin will start the menstrual flow in the non-pregnant, but would have no effect in the pregnant human. On this basis they proposed its use as a pregnancy test. This idea had its beginning in 1939 when Reynolds<sup>2</sup> reported that estrogens caused a transient increase in the amount of acetylcholine in the uterus of rats, and that the uterus thereby becomes hyperemic. Since that time several authors<sup>3-9</sup> have reported using prostigmin as a valuable test for pregnancy and treatment of 'nervous' amenorrhea in the absence of organic dysfunction. In a subsequent paper Taubenhau and Soskin<sup>10</sup> attempted to explain this effect of prostigmin from a study of the hypothalamic region and the release of luteinizing hormone. They offered evidence that prostigmin applied directly to the pituitary will cause a release of LH and a transient corpus luteum formation.

Since this drug is now commonly used it seemed desirable to know more about its

action on the reproductive system of the female. The present report deals with such a study in albino rats.

**Procedure and results.** Female rats of the Wistar strain were used. Prostigmin was injected twice daily. The daily dose for all small rats was .04 mg and for mature females 0.1 mg given in physiological salt solution in dilution of 0.1 mg of prostigmin per cc. Five groups of young female rats are shown in Table I. Group 1 consisted of a group of immature virgin females which weighed approximately 45 g at the start. The control animals received 1.5 units of gonadogen<sup>†</sup> daily, and the test animals also received the same amount of gonadogen plus 0.04 mg of prostigmin<sup>‡</sup> daily. Gonadogen was given in order that the immature uterus would be in a hyperemic state at the time prostigmin was given and would, therefore, be more like the premenstrual endometrium of the human than would the uterus of the untreated immature rat. After 5 days of treatment, the animals were sacrificed, the uteri and ovaries were weighed and desiccated for a period of 8 days, 4 days at 80°C followed by 4 days in a desiccator containing calcium chloride. The weights of these fresh organs are shown in Table I. Both ovaries and uteri are heavier in the prostigmin injected rats. Statistical analyses, using the mean difference divided by the standard error of the difference, are shown under "significant ratio" (S.R.) Any figure in this column (Table I) should be at least 3 in order to say that a significant change in weight has occurred between two means. In the present study ovaries and uteri of the prostigmin injected rats are compared with controls. In Group I the "significant ratio" was 2 for the ovaries and the same

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<sup>1</sup> Soskin, S., Wachtel, H., and Hechter, O., *J.A.M.A.*, 1940, **114**, 2091.

<sup>2</sup> Reynolds, S. R. M., *J. Physiol.*, 1939, **95**, 258.

<sup>3</sup> Grossmann, L. L., *W. J. Surg. Obst. and Gyn.*, 1942, **50**, 103; *ibid.*, 1944, **52**, 443.

<sup>4</sup> Carapetyan, H., *J.A.M.A.*, 1943, **122**, 81.

<sup>5</sup> Sneider, M. J., *Ill. Med. J.*, 1943, **83**, 107.

<sup>6</sup> Douglas, H. S., *W. J. Surg. Obst. and Gyn.*, 1943, **51**, 245.

<sup>7</sup> Friedmann, E., *Brit. Med. J.*, 1944, **1**, 11.

<sup>8</sup> Woodbury, R. A., Abreu, B. E., Torpin, R., and Fried, P. H., *J.A.M.A.*, 1945, **128**, 585.

<sup>9</sup> Parrella, D., *W. J. Surg. Obst. and Gyn.*, 1946, **54**, 397.

<sup>10</sup> Taubenhau, M., and Soskin, S., *Endocrinology*, 1941, **29**, 958.

<sup>†</sup> Gonadogen was kindly supplied by the Upjohn Company.

<sup>‡</sup> We are indebted to Hoffman-La Roche for the Prostigmin.

has been found to be an active hypotensive agent in hypertensive patients. Effective by mouth, it has produced fewer toxic side effects (nausea and vomiting), than any other active oral preparation presently available. This preliminary report summarizes our experience to date with this agent.

**Methods and Results.** The subjects were clinic and private cases of established essential hypertension. In 10 hospitalized patients the drug was given orally after a control period of at least 48 hours of bed rest. Measurements of arterial pressure and pulse rate were made by 2 independent observers at least 3 times a day, and (when these had stabilized) every half hour for the 2 hours before and 4 hours after the acute administration of the drug. The average (to the nearest 5 mm Hg) of the 4 measurements immediately before, and of the 4 lowest consecutive measurements after the administration of the drug were used to characterize its action.<sup>1</sup> In the 10 patients the average arterial pressures were lowered as follows: 220 125 to 155 90, 220 130 to 155 95, 220 140 to 135 90, 190 100 to 150 90, 190 125 to 160 110, 190 125 to 180 115, 195 100 to 170 95, 230 140 to 200 115, 190 125 to 165 110, and 240 130 to 190 110 mm Hg. In only 3 patients was there nausea, and in only 1 of these vomiting.

In 25 ambulatory cases at least 5 measurements were made of arterial pressure and pulse rate under standard conditions (sitting and/or lying) during each visit. After suitable oral observations the patient was instructed initially to take 1, or (in large persons) at most 2, mg of Veriloid 4 times a day at intervals of at least 4 hours (after each meal and at bedtime), unless this caused nausea, in which case he was to reduce the next dose, or if still nauseated to omit it entirely. After

1 week he was to return for a check-up, at which time, unless satisfactory results had already been achieved, he was instructed gradually to increase the dosage by not more than 1 mg a day (provided this caused no symptoms), adding 0.5 to 1 mg first to the bedtime, then to the after-breakfast, the after-lunch, and the after-supper doses, in that order, and to return for weekly checkups.

In this way, patients were gradually worked up to their nauseating dose, and then maintained at a slightly reduced dosage which averaged 2 mg of Veriloid 4 times a day at the end of 3 to 4 weeks. Patients were repeatedly cautioned, if nauseated or otherwise uncomfortable, to remain recumbent and to reduce slightly their subsequent doses. On this regimen, severe collapse reactions were rare after Veriloid. That definite toxic reactions could occur had been discovered during acute trial of the drug given in single doses of 4 to 6 mg. The antidote used for these reactions was the same as for those after other Veratrum preparations, namely, ephedrine sulphate intramuscularly (30 to 45 mg) and/or atropine (0.5 to 1 mg). Similar, but milder reactions occasionally occurred in patients on continued administration. Characteristically, these appeared only after several weeks of treatment and required a further small reduction of dosage. However, in only 5 patients was it necessary to discontinue the drug altogether.

Of 25 patients maintained to date on 6 to 12 (average 8) mg of Veriloid a day, 9 were followed for 12 to 20 weeks and had their average arterial pressures lowered as follows: 245 155 to 190 100, 250 150 to 210 110, 235 155 to 185 115, 235 150 to 230 135, 225 140 to 180 90, 180 120 to 140 85, 175 140 to 160 90, 160 115 to 160 95, and 190 110 to 155 90 mm Hg. Eleven patients took the drug for 6 to 12 weeks and had their average pressures changed from: 200 160 to 150 90, 160 100 to 160 80, 210 130 to 140 105, 150 140 to 170 80, 160 135 to 155 110, 210 140 to 180 90, 235 135 to 195 105, 215 130 to 170 95, 210 130 to 195 100, 195 110 to 180 110, and 165 90 to 155 80 mm Hg. Five patients were followed for 2 to 4 weeks and had pressures changed as

<sup>1</sup> The method of acute administration of the drug will be reported in detail later.

<sup>2</sup> Williams, R. W., Stanton, A. R., and Fries, E. Data to be published.

A meal should be well as usual. Patients should be free of nausea when food is taken in the stomach and on the subject is lying down. However, if food is taken within 3 hours after the drug, nausea is liable to occur.



groups received 0.1 mg of prostigmin daily.

Although the ovarian weights were unchanged in the seven day group and only slightly larger in those injected thirty days there was an increase in the percentage weight of the uteri in both Groups (4 and 5, Table I) which showed an analysis to have a "significant ratio" of 3 and 1.5 in Groups 4 and 5 respectively (Table I). Since uterine weights are more variable than those of the ovaries these "significant ratios" for the uteri are all the more interesting.

Pathology-histological examination<sup>§</sup> showed no consistent change in the ovaries. The uteri of the castrated prostigmin rats (Group 3) show the most changes; there were more round cells, edema and inflammatory cells, both polynuclear leucocytes and lymphocytes, than in the controls. The changes were almost completely confined to the submucosa. In the castrated controls both polynuclear leucocytes and lymphocytes are present but not numerous. Some edema and inflammatory cells were found in the submucosa cell groups including the controls. No changes in epithelial or musculature structure were observed in the prostigmin injected rats. The difference between the prostigmin and control animals is minor and pathologically there is no marked alteration.

**Discussion.** The ovaries of prostigmin injected rats were apparently in good condition as seen histologically and by the estrous

cycles. Twenty additional rats injected twice daily with 0.5 mg of prostigmin at each injection showed no significant change in estrous cycles as compared to saline injected controls. We were impressed by the fact that when the background was a mature uterus, induced by age as in Groups 4 and 5, or in hyperemic state brought about by estrogens Groups 1 and 3, prostigmin further increased the weight. This is also evident after corrections are made for differences in body weight and after desiccation. Thus in Group 3 the uteri in the prostigmin injected rats were 8.8, 9.9 and 9.2 percent heavier in the uncorrected, corrected for body weights and desiccated tissues respectively. This clearly shows that the increase in weight of the uterus after prostigmin was not due to edema.

**Summary.** Prostigmin was given to young rats in daily amounts of 0.04 to 0.1 mg. The ovaries were slightly increased in weight. The uteri if mature as the result of age or estrogens showed increase in weight following injections of prostigmin and these changes were present after desiccation. Estrous cycles showed no significant change from normal. Cellular elements of inflammatory cells were more abundant in the submucosa after prostigmin. On the whole, pathological changes were not marked; therefore, from the pathological viewpoint effects of prostigmin are minor as far as changes in the ovaries and uterus are concerned.

<sup>§</sup> Histological and pathological studies were kindly made by Doctor A. Nettleship of the Pathology Department.

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## Essential Hypertension. Therapeutic Trial of Veriloid, a New Extract of *Veratrum viride*.<sup>\*</sup> (17415)

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Veriloid (Coe Chemical Company), a purified alkaloidal fraction of *Veratrum viride*,<sup>1</sup>

<sup>\*</sup> Supported in part by Grants-in-Aid from the Coe Chemical Company, and the Squibb Institute for Medical Research.

<sup>†</sup> Present address: Georgetown University Medical Center, Washington, D.C.

<sup>1</sup> Stutzman, J. W., Maisson, G. L., and Kussnerow, G. W., *Proc. Soc. Exp. Biol. and Med.*, 1949, 71, 725.

TABLE I.  
Acute Toxicity of Di-(p-chlorophenyl)methylcarbinol for Rats and Mice Following Oral Administration by Stomach Tube.

Dose, mg/kg	Rats		Mice			
	Oil solution	Aq. suspension	Oil solution		Aqueous suspension	
	Mortality ratio		Paralysis ratio	Mortality ratio	Paralysis ratio	Mortality ratio
100			0/5	0/5	0/5	0/5
175			0/5	0/5	0/5	0/5
250	0/5	0/5	0/5	0/5	2/5	0/5
375	2/5	1/5	1/5	0/5	1/5	0/5
500	2/5	0/5	1/5	0/5	2/5	0/5
750	2/5	3/5	4/5	1/5	4/5	1/5
1000	2/2	4/9	4/5	1/5	5/5	3/5

TABLE II.  
Effect of a Diet Containing DMC on Weight and Ultimate Survival of Young Albino Rats.

% DMC in diet	Avg wt in g (10 rats per group) Time in days								
	0	5	10	19	26	35	42	63	70
.0	63.6	80.0	84.6	112	140	171	194	248 SE = 8.3	262 SE = 8.4
.1	63.6	73.7	85.8	113	140	176	194	226 SE = 6.5	245 SE = 7.8
.25	63.8	70.2	77.4	102(7)*	127(7)	172(4)†	177(4)	206(4)	220(4)
1.0	63.7	All dead after 72 hr							

\* 3 animals dead between 10th and 19th days.

† 3 animals sacrificed 28th day.

a moderate degree of hyperemia of the peritoneum and the abdominal viscera.

Mice survived the oral administration of 500 mg of DMC per kg, both as an oil solution and as an aqueous suspension, while dosages of 750 or more mg per kg resulted in the death of one or more of the 5 animals in the dosage group. During a period of 12 to 72 hours prior to death, mild to severe motor impairment of the posterior extremities was seen. The front limbs also were involved in a few cases. The motor signs rarely developed in less than 12 hours after administration of DMC. Recovery, rather than death, followed in some cases, a situation in sharp contrast to that seen in rats.

*Subacute Toxicity.* Four groups of 10 young female albino rats were fed, *ad libitum*, a powdered diet<sup>†</sup> containing 1.0, 0.25, 0.1, and 0.0% of DMC, respectively. The individu-

ally-housed animals were inspected daily, and were weighed periodically, but no attempt was made to measure food consumption. The progress of these animals is recorded in Table II.

The animals receiving the highest level of DMC (1.0%) became anorexic after 24 hours. Mild depression characterized their subsequent behavior and death occurred within 72 hours. The disturbances of locomotion and equilibrium seen after large single doses of DMC were not observed here. Autopsies performed immediately after death revealed hyperemia of the abdominal viscera as the sole gross finding.

The rats given the intermediate level of DMC (0.25%) consumed noticeably less food than the controls during the first 5 days. The intake of 7 of these animals increased and grossly equalled that of the controls during the next 5 days. The remaining 3 instead

<sup>†</sup> General Mills "Checkers."

follows: 260/150 to 190/110, 180/110 to 140/90, 200/115 to 180/110, 190/115 to 170/100, and 200/115 to 170/100 mm Hg.

Thus, on chronic as well as acute administration Veriloid was an active hypotensive agent, clinically similar to other *Veratrum* preparations previously tested.<sup>3-5</sup> Relative to its hypotensive effects, however, it appeared to produce somewhat less nausea and vomiting, the chief symptoms found in previous experience to interfere with the long-term usage of *Veratrum viride*. As with other preparations, meticulous adjustment of dosage to patient was the most important single point in the

chronic use of Veriloid. Patients objected so strongly to the symptoms associated with overdosage that it was the practice in the present study to be satisfied with moderate lowerings of arterial pressure, rather than to attempt dramatic effects at the expense of even occasional unpleasant side reactions.

**Summary.** In hypertensive patients Veriloid is an active hypotensive agent when administered acutely, or chronically for periods as long as 5 months. Meticulous regulation of dosage is important in obtaining optimum results. After several weeks of continuous therapy it is frequently necessary to reduce the dosage in order to avoid nausea and vomiting. Nevertheless, relative to its hypotensive effects Veriloid seems to produce fewer toxic reactions than any other oral preparation of *Veratrum viride* presently available.

<sup>3</sup> Freis, E. D., and Stanton, J. R., *Am. Heart J.*, 1948, **36**, 723.

<sup>4</sup> Freis, E. D., Stanton, J. R., Culbertson, J. W., Litter, J., Halperin, M. H., Burnett, C. H., and Wilkins, R. W., *J. Clin. Invest.*, 1949, **28**, 353.

<sup>5</sup> Wilkins, R. W., Freis, E. D., and Stanton, J. R., *J.A.M.A.*, 1949, **140**, 261.

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## Oral Toxicity of a New Miticide, Di-(p-chlorophenyl)methylcarbinol.\* (17416)

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Di-(p-chlorophenyl)methylcarbinol (DMC) synthesized by Bergmann and Bondi,<sup>1</sup> and later by Grummitt *et al.*,<sup>2</sup> has been shown to possess marked miticidal properties.<sup>3</sup> A preliminary study of its acute and subacute toxicity for rats and mice, following oral administration, is the subject of this report.

**Acute Toxicity.** Young albino rats (80-100 g) and adult albino mice (18-22 g), of the female sex, were given single doses of DMC by stomach tube. Solutions in corn oil and

suspensions in aqueous mucilage of tragacanth (2%) were used. Mice were observed for 10 days, and rats for 21 days, following intubation. The results of these experiments are shown in Table I.

With the oil solution of DMC the largest single dose tolerated by all 5 rats in a dosage group was 250 mg/kg. With the aqueous suspension one animal died following a dose of 375 mg/kg, although no fatalities resulted from dosage with 500 mg/kg. Fatalities from larger doses usually occurred 48 to 96 hours after administration. Urinary incontinence, tremors, ataxia, and impairment of righting reflexes were present for approximately 24 hours before death. Such abnormalities were not seen in animals which survived. Autopsies performed immediately after death revealed no gross pathological changes beyond

\* This study was supported by a grant-in-aid from the Sherwin-Williams Co., Cleveland, Ohio.

<sup>1</sup> Bergmann, E., and Bondi, A., *Berichte*, 1931, **64B**, 1455.

<sup>2</sup> Grummitt, O. J., Buck, A., and Becker, E., *J. Am. Chem. Soc.*, 1945, **67**, 2265.

<sup>3</sup> Witman, E. D., and Waters, H. A., unpublished observations.

have noted that the administration of female sex hormones cause a hyperactivity of the thyroid.<sup>11-17</sup> Investigations at present show that estrogenic substances exert an inhibitory influence on the structure and size of the testes and their accessories.<sup>5,18-25</sup> Some workers have obtained opposite results.<sup>10,26</sup>

The present investigation was undertaken because of the conflicting results that have been obtained.

**Materials and methods.** Three groups of male albino rats were used. Eight male rats of Group 1 (40 days of age) were castrated 2 weeks after the descent of the testes into the scrotum. Treatment began the day following castration and consisted of daily inunction of 270 rat units<sup>†</sup> of alpha-estradiol<sup>‡</sup>

in ointment form to the skin surface of the interscapular region. Group 2 consisted of eight, 15 day old normal non-castrated immature male rats. Treatment was carried out as in Group 1 but was started before the descent of the testes into the scrotum. Six rats in Group 3 served as control animals and received no treatment. On the day following the 24th application of alpha-estradiol on the experimental rats all the animals were killed with chloroform. The thyroids and testes were fixed in Bouin's fluid and the sections were stained with Ehrlich's acid hematoxylin and eosin. Klein's method<sup>27</sup> was employed for studying the size of the thyroid follicles and seminiferous tubules. The height of the follicular epithelium was determined by the method of Rawson and Starr.<sup>28</sup> An estimated percentage of destruction of the follicular walls was made by comparison with the findings of Morrell and Hart<sup>25</sup> who determined in percentage, the area of a section showing destruction. One hundred follicles from a section of the thyroid of each animal were studied for the number of follicles containing wall vacuoles in the colloid as described by Möllendorff.<sup>29</sup> The average number of primary and secondary spermatocytes was taken from 20 typical tubules in a section from each animal. The testes were compared as to gross size and as to the interstitial elements of Groups 2 and 3.

**Observations.** Thyroid follicular size and epithelial height were used to determine an increase or a decrease in thyroid activity. The results are shown in Table I. The long diameter of the thyroid follicles of Groups 1 and 2 averaged respectively 113.8 and 96.1  $\mu$ , while in the normal rats (Group 3) it was noticeably less with an average of 66.9 micra. The height of the follicular cells in Groups 1 and 2 averaged respectively 15.3 and 15.6  $\mu$ , while group 3 averaged 34.5  $\mu$ . Due to the

† Each application consisted of 0.15 g of ointment which contained 0.0225 mg of crystalline estradiol having a rat unit value of 270.

‡ Klein, J., *Ann. Int. Med.*, 1935, 8, 798.

28 Rawson, R. W., and Starr, P., *Arch. Int. Med.*, 1938, 61, 726.

29 Möllendorff, W., *Handbuch Mikroskop. Anat. des Mensch*, 1939, 6, 61.

<sup>7</sup> Costantini, P., *Rass. di Clin. Ter. e Sc. Aff.*, 1929, 10, 718.

<sup>8</sup> Sherwood, T. C., *Endocrinology*, 1940, 26, 693.

<sup>9</sup> Sherwood, T. C., *Endocrinology*, 1941, 29, 215.

<sup>10</sup> Korenchewsky, V., and Dennison, M., *J. Path. and Bact.*, 1935, 12, 323.

<sup>11</sup> Kunde, M. M., et al., *Proc. Soc. Exp. Biol. and Med.*, 1930, 28, 122.

<sup>12</sup> Benazzi, M., *Boll. Soc. Ital. Biol. sper.*, 1933, 8, 790.

<sup>13</sup> Leiby, G. M., *Proc. Soc. Exp. Biol. and Med.*, 1933, 31, 15.

<sup>14</sup> Pineus, G., and Werthessen, N., *Am. J. Physiol.*, 1933, 103, 631.

<sup>15</sup> Amilibis, E., et al., *Klin. Wochen.*, 1936, 15, 1901.

<sup>16</sup> Nathanson, I. R., et al., *Proc. Soc. Exp. Biol. and Med.*, 1940, 43, 737.

<sup>17</sup> Alexin, M., *Arch. f. Gyn.*, 1940, 159, 432.

<sup>18</sup> Golding, G. L., and Ramirez, F. T., *Endocrinology*, 1939, 12, 804.

<sup>19</sup> Burrows, H., *J. Path. and Bact.*, 1936, 42, 161.

<sup>20</sup> Greep, R. C., et al., *Anat. Rec.*, 1936, 65, 261.

<sup>21</sup> Gardner, W. U., *Anat. Rec.*, 1937, 68, 339.

<sup>22</sup> Ringo, A. R., *Anat. Rec.*, 1938, 72, 118.

<sup>23</sup> Pfeiffer, C. A., and Gardner, W. U., *Anat. Rec.*, 1938, 72, 59.

<sup>24</sup> van Wagenen, G., *Anat. Rec.*, 1935, 73, 387.

<sup>25</sup> Morrell, J. A., and Hart, G. W., *Endocrinology*, 1941, 19, 995.

<sup>26</sup> Allanson, M. J., *Exp. Biol.*, 1931, 8, 359.

<sup>†</sup> The author wishes to thank Dr. W. H. Stoner of the Schering Corporation at Bloomfield, N. J., for supplying the alpha-estradiol (Progynon D-H) used in this experiment.

became mildly depressed and completely anorexic. They died between the tenth and nineteenth experimental days. The survivors remained normal in appearance and behavior. Three were sacrificed on the twenty-eighth day and the remaining 4 at the end of the experiment. Mild hyperemia of the peritoneum and abdominal viscera was the only gross finding at autopsy in all 10 animals. There was no gross difference in average body weight between the few survivors and the controls.

The third group of rats whose diet contained 0.1% DMC showed a slight, though not significant, weight lag on comparison with the control group at 5 days. Between this time and the ninth week the average weights of the two groups were almost equal. Statistical analysis of the difference of 22 g between the average weights at 9 weeks yielded a critical ratio† of 2.2 which borders on significance. One week later the weight difference was 17 g and the ratio was only 1.5. Since the experiment was terminated at this time it is impossible to say whether these differences heralded the beginning of a significant lag in weight gain, and other signs of toxicity, in the treated group. The appearance and be-

havior of the two groups of rats did not differ in any way. Autopsies performed when the animals were sacrificed revealed no gross evidence of pathological changes in any organs or tissues.

*Comment.* These studies reveal that the toxicity of DMC is of the same order as that demonstrated by Woodard *et al.*<sup>4</sup> for DDT under grossly similar conditions. However, a more extensive comparison of the two compounds in the same laboratory would be necessary to determine accurately the relative toxicity of the two compounds.

*Summary.* 1. Rats and mice were intubated with single doses of a new miticidal compound, di-(p-chlorophenyl)methylcarbinol (DMC). The effect of graded doses on ultimate survival is discussed and the ability of large doses to produce signs suggestive of nervous impairment is described.

2. A powdered diet containing 0.1% DMC was well tolerated by growing rats during a ten week period.

3. The results compare favorably with those obtained in a similar limited study of DDT in another laboratory.

<sup>4</sup> Woodard, G., Nelson, A. A., and Calvery, H. O., *J. Pharm. Exp. Therap.*, 1944, **82**, 152.

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$$\frac{M_1 - M_2}{\sqrt{SE^2_{M_1} + SE^2_{M_2}}}$$

## Effects of Inunction of Alpha-Estradiol on Testes and Thyroids of Albino Rats.\* (17417)

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A diverse opinion exists in regard to the effect that the administration of sex hormones has on thyroid activity. Many investigators have noted that the thyroid remains normal or shows a slight degree of hypo-activity after castration or the administration of female

sex hormones.<sup>1-10</sup> In contrast many workers

<sup>1</sup> Cheuke, S., *Endocrinology*, 1930, **14**, 12.

<sup>2</sup> Aron, M., and Benoit, J., *Compt. rend. de Soc. Biol.*, 1932, **100**, 923.

<sup>3</sup> Starr, P., and Bruner, R. C., *Proc. Soc. Exp. Biol. and Med.*, 1935, **33**, 465.

<sup>4</sup> Shumacker, H. B., and Lamont, A., *Proc. Soc. Exp. Biol. and Med.*, 1935, **32**, 569.

<sup>5</sup> Uotila, V. U., *Endocrinology*, 1940, **26**, 123.

<sup>6</sup> Zalesky, M., *Anat. Rec.*, 1935, **62**, 109.

\* This work was carried out at the Department of Anatomy and Physiology, at the University of Kentucky, Lexington, under the direction of Professor R. S. Allen.

TABLE II.  
Histological Changes Occurring in the Testes of Group II as Compared to Group III, Which Was Considered Normal.

Group		Location	Size of tubules (Long diam.) in $\mu$	Size of testes compared to controls (Group III)	Sustentacular cells compared to controls (Group III)	Spermatogonia	Mitosis of spermatogonia	Avg No. per 20 tubules		Spermatids	Spermatozoa	Degeneration of tubular walls
								No. of primary spermatocytes	No. of secondary spermatocytes			
Group II Non-castrated treated	1	*	116.4	$\frac{1}{2}$	++	§	§	21.7	18.0	—	—	—
	2	*	130.1	$\frac{1}{2}$	++	§	§	30.3	23.7	—	—	—
	3	*	121.9	$\frac{1}{2}$	++	§	§	19.0	24.0	—	—	—
	4	*	136.9	$\frac{1}{2}$	++	§	§	16.9	14.2	—	—	—
	5	*	116.4	$\frac{1}{2}$	++	§	§	12.0	5.0	—	—	—
	6	*	112.3	$\frac{1}{2}$	++	§	§	15.6	18.0	—	—	—
	7	*	149.0	$\frac{1}{2}$	++	§	§	12.9	28.3	—	—	—
	8	*	147.9	$\frac{1}{2}$	+	§	§	11.6	10.5	—	—	—
	Avg		128.8	$\frac{1}{2}$				17.5	17.7			
Group III Normal control	1	†	257.5		+	§	§	58.6	142.4	§	§	—
	2	†	325.3		++	§	§	73.1	169.3	§	§	—
	3	†	282.1		++	§	§	69.0	154.2	§	§	—
	4	†	239.7		++	§	§	87.6	193.4	§	§	—
	5	†	343.8		++	§	§	78.4	181.4	§	§	—
	6	†	304.8		+	§	§	62.0	149.3	§	§	—
	Avg		292.2					71.2	165.0			

\* Abdomen. † Scrotum. ‡ Normal. § Present. — Absent.

its gonadotropic secretion. The testes of Group 2 did not remain in the abdomen long enough for all of the germ cells to disappear from the tubules. Spermatogenesis was reduced to the point that spermatid formation was completely absent. Probably these changes in the tubules were due to the alpha-estradiol indirectly, by preventing testicular descent, and permitting cellular changes due to retention in the body cavity. There was no noticeable change in the interstitial elements. The gross size of the testes were half that of the control animals.

of 270 rat units of alpha-estradiol (Progynon D-H) to normal and castrated immature male rats produced noticeable changes in the thyroid glands and in the testes of the non-castrated males. The thyroid glands appeared, histologically, in a hypoactive state. They exhibited an increased colloid content and a low follicular epithelium and destruction to the follicles. The hypoactivity appeared greater in castrated rats than in the non-castrated. Descent of the testes in immature males was prevented by the alpha-estradiol.

Summary. Daily inunction for 24 days

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TABLE I.  
Histological Changes Occurring in the Thyroid of Groups I and II as Compared to Group III,  
Which Was Considered Normal.

Group		Long diameter of follicles, $\mu$	Height of follicular epithelium, $\mu$	Vacuolated colloid per 100 follicles (wall vacuoles)	Estimated % of follicu- lar damage
I Castrated and treated	1	90.7	16.9	16	25
	2	108.7	17.2	31	25
	3	113.9	16.7	28	40
	4	120.5	13.6	35	40
	5	103.1	15.4	15	25
	6	116.4	15.7	43	40
	7	134.6	14.1	23	10
	8	122.6	13.7	39	10
	Avg	113.8	15.3	28.7	26.8
II Non-castrated and treated	1	99.5	17.8	26	0
	2	94.1	17.8	20	0
	3	83.0	13.2	23	10
	4	101.3	15.1	15	10
	5	95.4	13.7	31	25
	6	89.4	13.5	23	25
	7	110.0	21.2	19	10
	8	96.2	13.0	16	10
	Avg	96.1	15.6	21.6	11.2
III Normal control	1	63.0	23.2	7	0
	2	64.5	25.3	6	0
	3	70.5	39.7	4	0
	4	67.1	27.7	2	0
	5	74.7	52.0	8	0
	6	62.0	39.1	3	0
	Avg	66.9	34.5	5	0

increased follicular size and the lower epithelium of Groups 1 and 2 as compared with the normal animals it is apparent that hypoactivity of the thyroid gland was produced by the inunction of alpha-estradiol. The marked difference between Groups 1 and 2 indicates a greater hypothyroid condition in Group 1. This might be explained by the presence of the testes in Group 2, causing a higher androgen concentration.

Because of the greater increase of wall vacuoles in the colloid of the thyroids in Groups 1 and 2 in contrast to the lesser number in Group 3, mention is made of them although no physiological significance is attached to them at present. The wall vacuoles according to Möllendorff are formed by the contraction of the colloid due to fixation. All thyroids of this series were fixed for the same length of time in Bouin's fluid.

Damage to the follicular walls was greater in the castrated and treated rats than in the non-castrated, treated ones and was lacking

in the normal. Morrell and Hart<sup>30</sup> report that the thyroids of male and female rats treated with stilbesterol show destruction of the follicles brought about by invasion of leucocytes which may result from the static condition of the gland. The destruction occurred in areas which appeared hyalinized in some cases and fibrous in others. The damage to the thyroid follicles that appeared in Groups 2 and 3 was similar to that of Morrell and Hart. Fibrosis was noticeable in all the damaged thyroids and the hyalinized areas appeared to be due to release of follicular colloid in the tissue spaces.

Alpha-estradiol prevented the descent of the testes into the scrota of the animals of Group 2 (Table II). According to Moore and Price<sup>31</sup> this is due to the inhibitory effect of estrogens upon the hypophysis by reducing

<sup>30</sup> Morrell, J. A., and Hart, G. W., *Endocrinology*, 1941, **20**, 769.

<sup>31</sup> Moore, C. R., and Price, D., *Proc. Soc. Exp. Biol. and Med.*, 1930, **28**, 38.

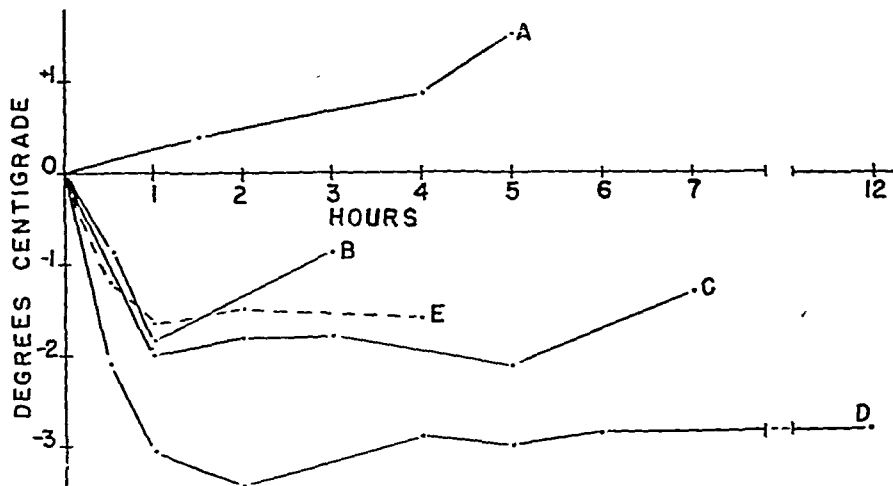


FIG. 1.

Average body-temperature changed after (1) subcutaneous injection (A) (15 rats) of 1.0 cc of Higgins India ink; (2) intraperitoneal injection of (B) 0.2 cc (8 rats), (C) 1.0 cc (8 rats), and (D) 2.0 cc (15 rats) of Higgins India ink, and (3) intraperitoneal injection of 2.0 cc of 2.0% suspension of blood charcoal (8 rats).

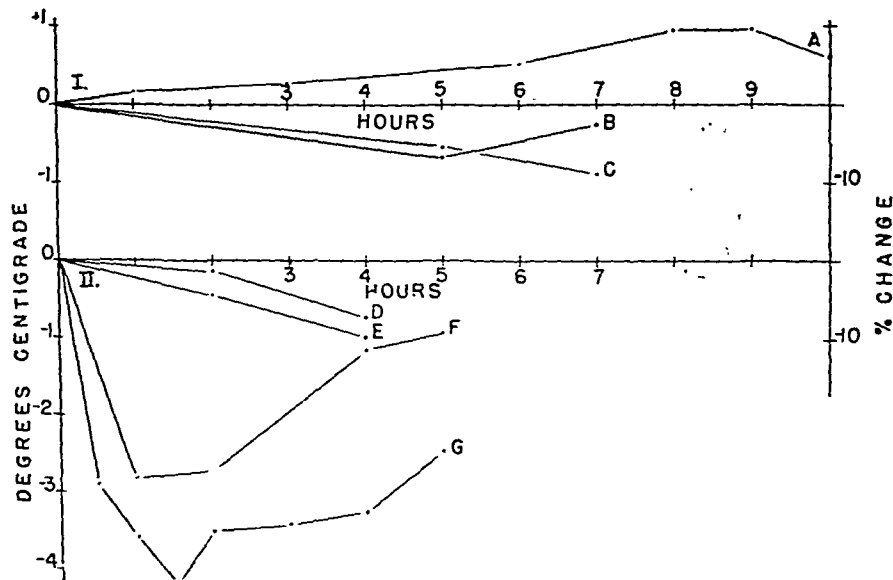


FIG. 2.

I. Average body-temperature change after subcutaneous injection of 4 cc of 1.0% carmine (A). Per cent change in cell volume (hematocrit) shown in C and plasma protein in B (15 rats).

II. Average body-temperature change after intraperitoneal injection of 4 cc (G) (8 rats) and 2 cc (F) (8 rats) of 1.0% carmine. Per cent changes in cell volume are shown in E and plasma protein in D for the rats of curve G.

injection. measurement of body-temperature and the care of the animals were the same as those already described.<sup>1</sup> Young adult white rats were used in all instances.

*Experimental Results.* Fig. 1 shows average

curves of body-temperature change after intraperitoneal administration of 0.2 cc (B), 1.0 cc (C), and 2.0 cc (D), and the subcutaneous administration of 1.0 cc (A) of India ink. In this figure is included a curve (E) that



## Effects of Injection of Large Molecular and Particulate Substances on Body-Temperature of Rats.\* (17418)

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In an earlier paper<sup>1</sup> it was shown that autolyzed yeast and the magnesium salt of yeast nucleic acid produce a rise in the body-temperatures of albino rats when injected subcutaneously, but produce a fall in the body-temperatures when injected intraperitoneally. On the other hand, the purines, pyrimidines and allantoin derived from nucleic acid do not materially affect body-temperature by either route of administration. It seemed possible, therefore, that the property of altering body-temperatures may be associated with the high molecular weight or particulate nature of the substances employed rather than with chemical groups present. India ink injected subcutaneously, as well as autolyzed yeast, has been used for producing fever experimentally. India ink is also known to be phagocytized after intraperitoneal injection and taken up by the cells of the reticuloendothelial system where it is easily seen. However, we have not found any record of body-temperature measurements after intraperitoneal injections of this substance.

In this paper we are reporting experiments with India ink, with blood charcoal and with two dyes that have been used for visualizing the lymph channels and the reticuloendothelial system, carmine and trypan blue. In addition to these, a variety of substances was tested either because of their particulate nature or, because they have been reported to alter body-temperature. In the latter group were skim milk, barium sulfate as prepared for gastrointestinal roentgenography, lipiodol, egg albumin, and 2, 4-dinitrophenol. Of these, only the last named showed any influence on body-temperature by either subcutaneous or intraperitoneal administration, and it pro-

duced fever by both routes. Because of the negative results no data are reported for this group of substances.

It seemed possible that the fall in body-temperature observed after intraperitoneal injection of some substances was a response to shock. To test this hypothesis blood cell volumes and plasma proteins were measured before and at intervals after treatment. These measurements are compared with similar measurements made after intraperitoneal injections of ox bile and solutions of peptone, two substances that are used for the experimental production of shock.

*Materials and methods.* The carmine used was a Grubler and Company preparation. The trypan blue was obtained from the Coleman and Bell Company. For most of the experiments both were prepared for injection by shaking 100 mg of the dye with 100 cc of 0.9% NaCl solution. Neither dye was completely soluble at this concentration, the carmine less so than the trypan blue, but both remained suspended long enough after vigorous shaking for injection. Except as indicated, Higgins India ink was used as purchased. Two g of Merck's blood charcoal were shaken vigorously with 100 cc of 0.9% NaCl solution just before injection. Ox bile was used as received from the packing house. The peptone was a 20% aqueous solution of J. T. Baker's powdered "Peptone, Bacteriological." Blood for cell volume and plasma protein determinations was obtained by clipping the tail and allowing the blood to drop into a small cuvette containing heparin. Cell volumes were determined with Smith tubes and plasma proteins by the copper sulfate specific gravity method.<sup>2</sup> The methods of

<sup>2</sup> Phillips, R. A., Van Slyke, D. D., Dole, V. P., Emerson, K., Hamilton, P. B., and Archibald, R. M., Publication of the United States Navy Research Unit at the Hospital of the Rockefeller Institute for Medical Research.

\* Supported under contract with the Office of Naval Research.

<sup>1</sup> Hill, R. M., and Rutledge, E. K., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 9.

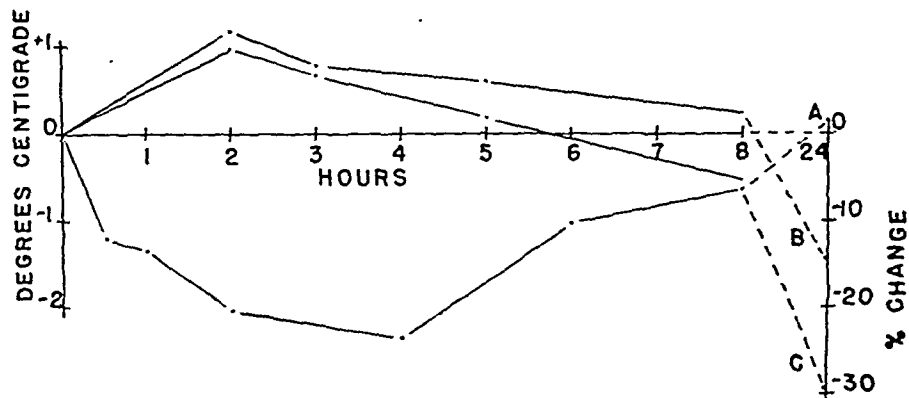


FIG. 4.

Average changes in body-temperature (A) after the intraperitoneal injection of 4 cc of 20% peptone. Per cent change in cell volume (hematocrit) (C) and plasma protein (B) in the same animals (6 rats).

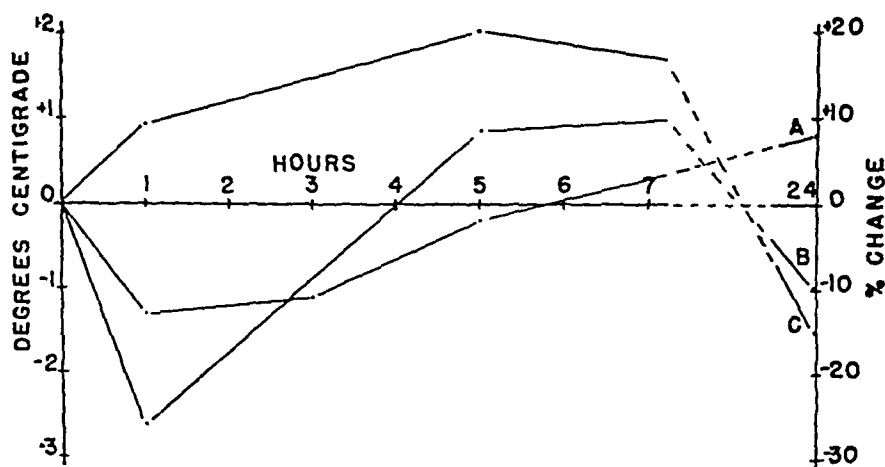


FIG. 5.

Average change in body-temperature (A) after the intraperitoneal injection of 0.5 cc of ox bile. Per cent change in cell volume (hematocrit) (C) and plasma protein (B) in the same animals (6 rats).

(16%) cannot be explained entirely on the basis of dilution. We suggest that the more concentrated carmine preparation contained enough insoluble material that local irritation resulted in mild shock. This, if it is shock, is accompanied, however, not by a more intense but less intense hypothermia.

To save space, no curves are given for the trypan blue experiments. In all cases they were qualitatively like those for carmine; quantitatively, the response was less intense.

Fig. 4 shows curves for body-temperature response and for changes in blood cell volume and plasma protein concentration after intra-

peritoneal injection of 4 cc of 20% peptone. The decrease in body-temperature (A) is similar to that after 1 cc of India ink or 1 cc of 1% carmine, but is not as great as that after 4 cc of either of these preparations. In spite of the large amount of water administered, there was an early concentration of the blood, as measured by blood cell volume (C) and plasma protein concentration (B), which was followed later by dilution. These changes may be due to an early loss of water into the peritoneal cavity because of hypertonicity of the peptone solution. The fact that the protein and cell volume changes were

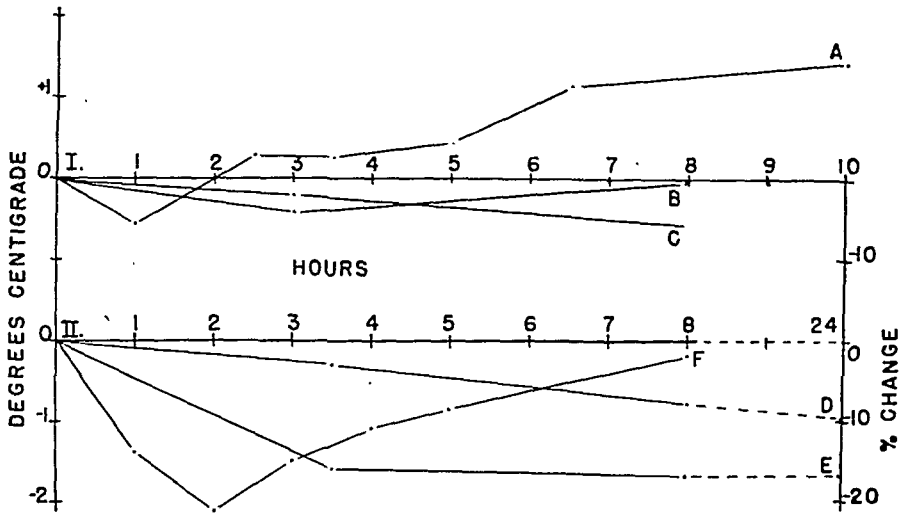


FIG. 3.

I. Average body-temperature change (A) after intramuscular injection of 1 cc of 4% carmine. Average per cent change in cell volume (hematoerit) (B) and plasma protein (C) in the same animals (4 rats).

II. Average body-temperature change (F) after intraperitoneal injection of 1 cc of 4% carmine. Average per cent change in cell volume (D) and plasma protein concentration (E) in the same animals (8 rats).

represents the changes in body-temperature after intraperitoneal administration of 2.0 cc of a 2.0% suspension of blood charcoal.

The expected rise in body-temperature was observed after subcutaneous injection of India ink. After intraperitoneal injection, the body-temperature showed a fall, the degree and duration of which depended upon the amount of ink administered. Dilutions of India ink 1-100 given in 2 cc doses were ineffective; 1-10 dilutions given in the same volumes gave small responses in some animals. These data are not represented in the figure. Blood charcoal given intraperitoneally gave less intense but qualitatively the same response as India ink.

Carmine (Fig. 2) in doses of 4 cc of a 1.0% preparation produced a fever when given subcutaneously (A) but was somewhat less active than 2 cc of India ink. The same dose given intraperitoneally (G) reduced the body-temperature, both qualitatively and quantitatively, much as was done by 2.0 cc of India ink. Intraperitoneal injection (F) of 2.0 cc of 1.0% carmine gave a less prolonged response. The changes in cell volume (subcutaneous injection C; intraperitoneal

injection E) and plasma protein concentration (subcutaneous injection B; intraperitoneal injection D) may have been caused by dilution with the water injected. Similar changes followed the injection of saline alone.

The results of intramuscular injection of carmine are shown in Fig. 3 (I). In order to avoid over-distention of the tissue and also to give the same dose of carmine, 1 cc of a 4% preparation was used instead of 4 cc of a 1% preparation. After a short period of hypothermia, a prolonged hyperthermia followed this treatment. The more mild decreases in cell volume and plasma protein concentration after injection of the smaller volumes are consistent with the hypothesis that these phenomena are due to dilution.

In Fig. 3 (II) are shown the results of intraperitoneal injection of 1 cc of the 4% preparation of carmine. This more concentrated preparation given in smaller volume has less effect on body-temperature (F) than 4 cc of the 1% preparation (Fig. 2). At the same time there is a greater fall in the plasma protein concentration (E) and a somewhat greater fall in the cell volume (D). The great fall in plasma protein concentration

animals were not in shock during hypothermia. Intraperitoneal injections of ox bile, and solutions of peptone produced a less intense

hypothermia although ox bile caused severe shock.

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## A Highly Active Material that Promotes Conversion of Prothrombokinase Complex.\* (17419)

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A procedure has been described<sup>1</sup> for analyzing blood coagulation in three experimental steps: 1. activation of prothrombokinase; 2. activation of prothrombin; 3. coagulation of fibrinogen. Crude thrombokinase, besides activating prothrombin, also accelerated conversion of prothrombokinase. Both activities were lost upon adsorption with barium sulfate.

It is now reported that a material, prepared by elution from barium sulfate, promotes the activation of prothrombin, and also the conversion of crude prothrombokinase. Beginning with a solution of bovine plasma globulins, fraction "A" was adsorbed on Hyflo (Johns-Manville) and eluted with phosphate-potassium chloride solution. The unadsorbed globulins were exposed to adsorption by barium sulfate. From the latter, two successive fractions were eluted, with 0.2-0.3M phosphate, pH 6.6, and with 0.6M phosphate, pH 7.9. The pH 6.6 eluate had more prothrombin. From the pH 7.9 eluate, proteins soluble in 0.35, but precipitable by 0.45 saturated ammonium sulfate were selected as the "converter fraction." This involved 3 extractions and 20 precipitations. Proteins soluble in 0.45, but precipitable by 0.60 saturated ammonium sulfate, constituted the "thrombin fraction" (3 extractions, 7 precipitations). Fifty-five liters of plasma yielded 10 ml of each fraction. Both were dialyzed simultaneously in the same beaker.

Activations were studied with calcium

present, giving results summarized in Table I. At high dilution, the converter hastened both the activation of prothrombin and the conversion of crude prothrombokinase. In comparing different preparations, converter activities estimated by the 2-stage and 3-stage procedures were roughly parallel. This is compatible with the view that the tests measured two different effects of the same substance.

In contrast, converter activity did not parallel thrombin activity. The ratio of converter to thrombin activity was 100 times as great in the converter fraction as in the thrombin fraction. Conceivably, varieties of thrombin can exist with different converter activities, or some factor modifies the behavior of thrombin in one of the fractions. If, as must be contemplated, the converter is distinct from thrombin, the results suggest how difficult would be its elimination from thrombin by salting-out. Moreover, the converter is manifest in sufficiently minute amount to cause confusion, even as a small contamination in a highly purified thrombin.

The relation of "A" and converter to thrombokinase activity resembles that reported for platelets plus "globulin".<sup>2</sup> Their effects are complementary, but not simply additive. Clear evidence has not been obtained that their mutual reinforcement is due chiefly to progressive activation of one by the other. An additional possibility must be considered, that one is thrombokinase and the other contains a co-factor or anti-inhibitor.

\* Aided by a grant from the James Hudson Brown Memorial Fund of the Yale University School of Medicine.

<sup>1</sup> Milstone, J. H., *J. Gen. Physiol.*, 1948, 31, 301.

<sup>2</sup> Milstone, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1948, 68, 225.

closely parallel argues against loss of protein into the tissue through the capillary wall. If shock was present in these animals during hypothermia, it was not severe. We cannot explain the dilution of the blood the following day. The animals appeared to be in good condition.

In Fig. 5 are presented the body-temperature changes (A) and changes in the blood cell volume (C) and plasma protein concentration (B) after intraperitoneal injections of 0.5 cc of ox bile. It was found impossible to administer more than this quantity of bile and keep the animals alive as long as was desired. Following bile administration there was a decrease in plasma protein concentration at the same time that the blood was being concentrated as measured by the cell volume. This can be explained as an early local loss of protein through the damaged capillary walls. At the site of protein loss there was presumably a parallel loss of water. In other areas the water was partly restored but the protein could not be supplied as quickly. The net result of loss of water and of protein in one area and partial replacement of water alone in another area is dilution of the circulating plasma protein and a simultaneous concentration of cells. However, later, the plasma protein concentration was increased, and, at the 5th hour was 8% above the initial value. Finally, there was a roughly parallel decrease in both the cell volume and the plasma protein concentration indicating a general dilution of the blood. At autopsy it was observed that engorgement was intense in the splanchnic veins. We believe that the development of shock after intraperitoneal injection of ox bile was unquestionable. However, in spite of the shock, the intensity and duration of the hypothermia was less than was found after injection of the other hypothermia-provoking agents. Gross examination of the peritoneal surfaces after injection of the substances other than bile showed at most only mild reactions.

*Discussion.* The substances, India ink, blood charcoal, carmine, and trypan blue, whose dual action on body-temperature is described in this paper, have little in common chemically but, as administered, all are

particulate. When given intraperitoneally, they are all rapidly taken up by the lymph channels and can be seen in the reticuloendothelial system. However, the fact that a substance is injected in particulate form does not assure that it will affect body-temperature. This is shown particularly well with injections of suspensions of  $\text{BaSO}_4$  which are entirely inactive. It may be that the particles of  $\text{BaSO}_4$  are not phagocytized and do not reach the reticuloendothelial system and that this is the reason for its inactivity. On the other hand, no particulate material can be seen in the clear yeast "autolysates" which have a dual effect on body-temperature in the rat as previously described.<sup>1</sup> The very large molecules of yeast nucleic acid, which are presumed to be the active constituent of yeast autolysate, may be handled similarly to particulate material, though we have, as yet, no evidence of this beyond the similarity in thermal reaction after injection.

At present, no explanation can be given for the fall in body-temperature after the intraperitoneal injection of these substances. There is little evidence of shock, and when severe shock is produced by intraperitoneal injection of ox bile, the fall in body-temperature is less than when particulate substances are injected. The cause of hypothermia would seem to lie rather in some interference with the normal functioning of the reticuloendothelial system. Intramuscular injection of carmine produces a rise in body-temperature similar to that following subcutaneous injection. This would seem to rule out differences in blood supply to the area of injection, and consequent differences in the rate of absorption, as the cause of the dual effect of carmine on body-temperature.

*Summary.* India ink, suspensions of blood charcoal, carmine, and trypan blue were injected subcutaneously and intraperitoneally. Carmine was injected also intramuscularly. After subcutaneous and intramuscular injection a rise in body-temperature was observed. After intraperitoneal injection a fall in body-temperature was observed. As judged by relative engorgement of capillary beds, and changes in blood cell volume (hematocrit) and plasma protein concentration, these

have found that the factor in the serum responsible for the agglutination of normal sheep erythrocytes was present in the sera of both normal and rheumatoid arthritic individuals. This normal agglutinin is a variable factor and is independent of the capacity of the sera of patients with rheumatoid arthritis to agglutinate sensitized cells. It can be selectively absorbed without significantly influencing the titer against sensitized sheep cells. The titer thus obtained reflects the concentration of the factor associated with rheumatoid arthritis. At the same time the need for expressing the results of the test as a differential titer is eliminated. Based upon an absorption technic, a modification of the original test is herein described. A comparison of the results of the test as modified by us, the test as originally described by Rose, *et al.*,<sup>1</sup> and the streptococcus agglutination test is presented.<sup>4-6</sup>

**Method and procedure.** 1. *Preparation of Normal Sheep Erythrocytes.* One volume of sheep blood is mixed with 1.2 volumes of Alsever solution. Sufficient formaldehyde is added at collection to make a final concentration of 0.2%. This mixture stored at 4-6°C will usually last 4 to 6 weeks. The blood-Alsever mixture should be kept in the refrigerator for 2-3 days before use. The cells are washed 3 times in a graduated centrifuge tube with saline, centrifuging at 2000 rpm for 15 minutes between each washing. The supernatant of the last washing must be water clear. The packed cells are used for absorption. A 2% suspension in saline is also prepared from packed cells. Part of this is further diluted with saline to make a 1% suspension for use in the test.

*Preparation of Sensitized Sheep Erythrocytes.* Rabbit anti-sheep-erythrocyte hemolysin is prepared in concentrations of 1:4000 (2 units per cc) determined by the complement-fixation technic.<sup>1</sup> To the 2% suspension of

erythrocytes as described above an equal volume of a 1:4000 dilution of amboceptor is added. The solution should be allowed to stand for at least 10 minutes before use.

*Treatment of patient's serum.* Clear serum is obtained from clotted blood and inactivated at 56°C for one-half hour. Serum may be stored in a deep freeze at -20°C for at least 5 months without significant loss of potency.<sup>7</sup> For absorption, 4 volumes of serum are mixed with 1 volume of packed sheep erythrocytes and allowed to remain at room temperature for 40 minutes with gentle shaking at intervals. The mixture is centrifuged and the clear serum is decanted into an additional volume of packed cells for another 40-minute incubation period at room temperature. The mixture is again centrifuged and the clear serum is used for the test. Two absorptions have been adequate to eliminate the normal agglutinating factor for normal sheep cells. Additional absorptive treatment is necessary in cases where the heterophile antibody associated with infectious mononucleosis is present. For this purpose, a 20% boiled ox erythrocyte suspension<sup>8</sup> is centrifuged and the supernatant fluid discarded. Four volumes of the serum is mixed with one volume of the packed sediment, incubated at 37°C for 1 hour, centrifuged, and the serum removed.

*Procedure.* Two series of two-fold dilutions of absorbed serum are made in 0.5 ml volumes of normal saline. In the first series, consisting of three tubes, the serum dilutions range from 1:3.5 to 1:14. To this series is added an equal volume (0.5 ml) of 1% normal sheep cells; the final dilution range is thus 1:7 to 1:28. The second series, consisting of 10 tubes, is similarly diluted (1:3.5 to 1:1792). To this series, 1% sensitized sheep cells are added in 0.5 ml volume, thus making the final dilutions 1:7, etc. The tubes are shaken to make a homogeneous suspension of the cells, and incubated in a water bath at 37°C for one hour. The tubes are then refrigerated overnight. Readings are made after gentle

<sup>7</sup> Unpublished data.

<sup>8</sup> Kolmer, J. A., and Boerner, F., *Approved Laboratory Technic*, D. Appleton-Century Co., N. Y., 4th ed., 1945, p. 644.

<sup>1</sup> Cecil, R. L., Nicholls, E. E., and Stainsby, W. J., *Arch. Int. Med.*, 1929, **43**, 571.

<sup>5</sup> Cecil, R. L., Nicholls, E. E., and Stainsby, W. J., *Am. J. Med. Sc.*, 1931, **181**, 12.

<sup>6</sup> Lipman, M. O., Laboratory of the Edward Daniels Faulkner Arthritis Clinic, Presbyterian Hospital, New York, personal communication.

TABLE I.  
Effective Amounts of Converter Fraction.

2-stage tests		3-stage tests
Activate half of prothrombin in 40 min.	Accelerate prothrombin activation in presence of aged fraction "A"	Accelerate conversion of crude prothrombokinase
2/11 dilution	1/1,000 dilution 1 $\mu$ g protein-N/ml	1/45,000 dilution 0.02 $\mu$ g protein-N/ml

Figures refer to final dilution or concentration in the respective activation mixtures. A concentration of 0.02  $\mu$ g protein-N per ml prothrombokinase mixture becomes 0.002  $\mu$ g per ml when the mixture is diluted tenfold in prothrombin. The tests were performed as previously described.<sup>1,2</sup>

Previous comparison<sup>1</sup> of prothrombokinase to Factor V was based on Owren's tentative concept of V as the precursor of VI.<sup>3</sup> If his V preparation did not so function, the comparison does not apply to it. Crude prothrombokinase "may contain an activator complex with more than one significant component."<sup>1</sup> More than one transformation may occur in the complex.

Although the converter fraction is highly active, it may be far from pure, chemically.

<sup>3</sup> Owren, P. A., *Acta Med. Scand.*, 1947, suppl. 194.

<sup>4</sup> Milstone, J. H., *J. Gen. Physiol.*, 1942, 25, 679.

It may represent a thrombokinase which activates not only prothrombin, but also prothrombokinase. Several years ago,<sup>4</sup> it was reported that concentrated prothrombin changed to thrombin in the absence of ionic calcium, without addition of activators; and the possibility was left open that a contaminant was responsible. The possibility must be entertained that the converter contaminates highly purified clotting preparations, and might be responsible for certain effects occasionally attributed to prothrombin or thrombin.

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## A Modification of the Hemagglutination Test for Rheumatoid Arthritis.\* (17420)

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A diagnostic test for rheumatoid arthritis based upon the agglutination of sheep erythrocytes has been recently reported by Rose, Ragan, Pearce and Lipman.<sup>1</sup> Serum from patients with rheumatoid arthritis agglutinates sheep erythrocytes sensitized with sheep

cell hemolysin to a titer at least 16 fold higher than that obtained with normal sheep cells. These are expressed as a differential titer  $\left( \frac{\text{titer with sensitized cells}}{\text{titer with normal cells}} \right)$ . The test has been confirmed but may be of limited value for diagnostic purposes.<sup>2,3</sup> In the performance of the test as described by Rose, *et al.*,<sup>1</sup> we

\* Published with the permission of the Chief Medical Director, Department of Medicine and Surgery, Veterans Administration, who assumes no responsibility for the opinions expressed or the conclusions drawn by the authors.

<sup>1</sup> Rose, H. M., Ragan, C., Pearce, E., and Lipman, M. O., *Proc. Soc. Exp. Biol. and Med.*, 1948, 68, 1.

<sup>2</sup> Sulkin, S. E., Pike, R. M., and Coggeshall, H. C., *Proc. Soc. Exp. Biol. and Med.*, 1949, 70, 475.

<sup>3</sup> Jawitz, E., and Hook, E. V., *Proc. Soc. Exp. Biol. and Med.*, 1949, 70, 650.

TABLE II.  
Differential Absorption of the Heterophile Factor from Serum of Patients with Infectious Mononucleosis and Patients with Rheumatoid Arthritis.

Diagnosis	Titer of unabsorbed serum +			Titer of serum absorbed with normal sheep cells +		Titer of serum absorbed with sheep and ox cells +	
	Normal RBC	Sensitized RBC	Differential titer	Normal RBC	Sensitized RBC	Normal RBC	Sensitized RBC
1. Infectious mononucleosis	896	1792	2	112	448	0	0
2. Rheumatoid arthritis	14	448	32	0	448	0	448
3. Mixture of equal vol. of No. 1 and 2	448	896	2	224	896	0	224
4. Infectious mononucleosis	896	896	1	112	448	0	0
5. Rheumatoid arthritis	28	3584	128	0	1792	0	1792
6. Mixture of equal vol. of No. 4 and 5	448	896	2	112	1792	0	1792

RBC refers to sheep red blood cells.

arthritis (control group) gave no significant agglutination. Removal of the normal (non-specific) factor by absorption did not materially affect the titer of the factor associated with rheumatoid arthritis. The sera from cases of infectious mononucleosis could not be completely absorbed by the routine method described. The heterophile antibody continued to give a positive titer with normal sheep cells after absorption of the sera with sheep erythrocytes. However, additional absorption of the sera with boiled ox cells resulted in complete absorption of the heterophile factor. In cases of active peripheral rheumatoid arthritis, such multiple absorptions did not affect the final titer. Mixtures of sera from cases of rheumatoid arthritis and cases of infectious mononucleosis, when treated by this method and allowing for dilutions, retained only the titer of the rheumatoid arthritis factor (Table II).

Table III shows the distribution of agglutination titers using the modified test in 166 cases. Only cases with active peripheral rheumatoid arthritis (with the exception of 2 cases of infectious hepatitis) gave titers of 1:28 or higher with complete agglutination in the first 2 dilutions. For this reason, this value is considered to be the lower limit of a positive reaction. Using this criterion, 35 of 39

cases (90%) of active peripheral rheumatoid arthritis (8 of which also had spondylitis) gave positive values ranging from 1:28 to 1:3584. All 30 were unequivocal cases of active peripheral rheumatoid arthritis with x-ray evidence of small joint involvement. Negative reactions were obtained with the sera of all cases of inactive<sup>†</sup> peripheral rheumatoid arthritis, rheumatoid spondylitis without peripheral joint involvement or with inactive peripheral joint involvement, and in all cases of psoriatic arthritis. A group of 13 cases of arthritis in which the clinical course was not inconsistent with the picture of early peripheral rheumatoid arthritis but in which x-rays showed no evidence of joint involvement were all negative. All other forms of arthritis, non-rheumatoid in type, and the non-arthritic controls were negative. Ten cases of infectious hepatitis were tested. No determination could be made in one of these cases by our method because complete absorption could not be obtained. After repeated absorptions (10 times), the titer against unsensitized cells remained 1:28. In two other cases the titers were positive (Table III).

<sup>†</sup> A case was considered inactive if the patient was clinically in symptomatic remission with absence of swelling, pain and tenderness of involved joints and with the sedimentation rate normal.



TABLE I.  
Comparison of Agglutination Titers Obtained with Unabsorbed and Absorbed Sera from Representative Cases.

Diagnosis	Rose test			Modified test	
	Unabsorbed serum + Normal RBC	Sensitized RBC	Differential titer	Absorbed serum + Normal RBC	Sensitized RBC
Active peripheral rheumatoid arth- ritis (5 cases)	56	896	16	0	896
	56	1792	32	0	1792
	0	448	448	0	224
	14	112	8	0	224
	56	448	8	0	448
Rheumatoid spondy- litis with active peripheral joint involvement (4 cases)	112	224	2	0	112
	0	896	896	0	1792
	14	1792	128	0	896
	0	896	896	0	1792
Rheumatoid spondy- litis without peripheral joint involvement (3 cases)	7	7	1	0	0
	14	28	2	0	0
	28	56	2	0	0
Psoriatic arthritis (2 cases)	14	14	1	0	0
	7	14	2	0	0
Cirrhosis of liver	56	112	2	0	0
Hyperglobulinemia (2 cases)	14	14	1	0	0
	28	56	2	0	0
Brucellosis	7	14	2	0	0
Duodenal ulcer	0	14	14	0	0
Gout	14	28	2	0	0
Osteoarthritis	7	28	4	0	0
Rheumatic polyarthritis	28	28	1	0	0

RBC refers to sheep red blood cells.

shaking. For simplicity, 3 values are used: complete agglutination, partial agglutination, and no agglutination perceptible to the naked eye. The test is valid only if no agglutination is observed in the tubes containing the normal cells, thereby indicating complete absorption of the normal sheep cell agglutinin. Both the Rose test and the modified test were run concurrently; the same reagents were always used for both tests. The Rose test was performed as described<sup>1</sup> except that the serial dilutions were the same as those used in our modified test. The streptococcus agglutination test was also performed as described by Lipman.<sup>6</sup>

*Results.* Agglutinins for normal sheep erythrocytes (Forssman antibody) may be

found in the sera of normal individuals as well as in those with rheumatoid arthritis. In our studies, it was found that sera obtained at intervals from the same patient showed variations in the normal agglutinin titer.<sup>7</sup> It is not yet clear whether these variations reflect a fluctuating agglutinin content or are associated with the varying agglutinability of different batches of sheep erythrocytes. Table I demonstrates that this normal agglutinin can be completely absorbed with normal sheep cells. As a result of this procedure, only the sera from patients with active peripheral rheumatoid arthritis retained the capacity to agglutinate sensitized cells. The sera from patients with diseases other than rheumatoid

TABLE II.

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Diagnosis	Titer of unabsorbed serum +			Titer of serum absorbed with normal sheep cells +		Titer of serum absorbed with sheep and ox cells +	
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RBC refers to sheep red blood cells.

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Table III shows the distribution of agglutination titers using the modified test in 166 cases. Only cases with active peripheral rheumatoid arthritis (with the exception of 2 cases of infectious hepatitis) gave titers of 1:28 or higher with complete agglutination in the first 2 dilutions. For this reason, this value is considered to be the lower limit of a positive reaction. Using this criterion, 35 of 39

cases (90%) of active peripheral rheumatoid arthritis (8 of which also had spondylitis) gave positive values ranging from 1:28 to 1:3584. All 30 were unequivocal cases of active peripheral rheumatoid arthritis with x-ray evidence of small joint involvement. Negative reactions were obtained with the sera of all cases of inactive<sup>†</sup> peripheral rheumatoid arthritis, rheumatoid spondylitis without peripheral joint involvement or with inactive peripheral joint involvement, and in all cases of psoriatic arthritis. A group of 13 cases of arthritis in which the clinical course was not inconsistent with the picture of early peripheral rheumatoid arthritis but in which x-rays showed no evidence of joint involvement were all negative. All other forms of arthritis, non-rheumatoid in type, and the non-arthritic controls were negative. Ten cases of infectious hepatitis were tested. No determination could be made in one of these cases by our method because complete absorption could not be obtained. After repeated absorptions (10 times), the titer against unsensitized cells remained 1:28. In two other cases the titers were positive (Table III).

<sup>†</sup> A case was considered inactive if the patient was clinically in symptomatic remission with absence of swelling, pain and tenderness of involved joints and with the sedimentation rate normal.

TABLE III.  
Distribution of Cases According to Agglutination Titer, Using the Modified Test.

Diagnosis	No. of cases	Agglutination titer											
		0	7	14	28	56	112	224	448	896	1792	3584	
Active peripheral rheumatoid arthritis													
Without spondylitis	31			1	2(a)	2	3	4	8	5	5	1	
With spondylitis	8	2			2		1			1	2		
Peripheral rheumatoid arthritis of questionable activity (1)	7	3		2	1(b)	1							
Inactive peripheral rheumatoid arthritis	8	5	1	1	1(b)								
Rheumatoid spondylitis without peripheral joint involvement	15	12	1	2									
Rheumatoid spondylitis with inactive peripheral joint involvement	7	5	2										
Psoriatic arthritis	9	6	1	2									
Arthritis, type undetermined, possibly rheumatoid (2)	13	10	2	1									
Other arthropathies (3)	30	27	2	1									
Non-arthritic controls except infectious hepatitis (4)	28	23	3	1	1(b)								
Infectious hepatitis	10(c)	3	2	2	1	1							

(1) Definitive evaluation of activity could not be made on the basis of clinical impression and laboratory data.

(2) Clinical course was not inconsistent with early rheumatoid arthritis, but x-ray studies showed no changes in affected joints.

(3) These include 10 cases of rheumatic polyarthritis, 7 cases of gout, 2 cases of osteoarthritis, and 11 cases of arthritis following a variety of infection.

(4) Among these are included 3 cases of hyperglobulinemia and 4 cases of infectious mononucleosis.

(a) One of these is considered negative because the results showed incomplete agglutination in first two tubes.

(b) Negative because of incomplete agglutination in first two tubes.

(c) Determination of titer in 1 case of infectious hepatitis could not be made because of incomplete absorption of the non-specific factor by the standard technic.

Table IV presents a summary of the results obtained with the sera of 166 patients by the modified test, the original Rose technic, and the streptococcus agglutination test. In the group of 39 patients with active peripheral rheumatoid arthritis of varying severity and duration, eight of whom also had spondylitis, the modified test gave a significantly higher percentage of positive reactions than either of the other two tests. Both the modified and the Rose tests were consistently negative in inactive peripheral rheumatoid arthritis, active or inactive spondylitis without peripheral joint involvement, and in psoriatic arthritis. In these groups, the streptococcus agglutination test, however, was found on several occasions to be positive. The group of 13 cases of arthritis in which no definitive diagnosis could

be made were negative to all 3 tests employed. In all other arthropathies, including osteoarthritis, rheumatic polyarthritis, gout, *etc.*, the modified test and the Rose test were consistently negative. This was also true of the non-arthritic controls with the exception of infectious hepatitis in which two were positive by our test but negative by the Rose test.

Table V presents the findings in the 11 cases of active peripheral rheumatoid arthritis in which the Rose test was negative whereas the modified test was positive. It is to be noted that in each case the titer against the sensitized sheep red cells was significantly elevated but the differential titer in the Rose test fell below 16 because of the presence of a relatively high non-specific agglutinin titer. This non-specific factor was removed in the

TABLE IV.  
Summary of Results Comparing Tests Employed.

Diagnosis	No. of cases	Modified test (1)		Rose test (2)		Streptococcus agglutination	
		Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Active peripheral rheumatoid arthritis							
Without spondylitis	31	29	2	21	10	18	13
With spondylitis	8	6	2	3	5	5	3
Total	39	35	4	24	15	23	16
%	(100)	(90)	(10)	(61)	(39)	(58)	(42)
Peripheral rheumatoid arthritis-questionable activity	7	1	6	1	6	2	5
Inactive peripheral rheumatoid arthritis	8	0	8	0	8	2	6
Rheumatoid spondylitis without peripheral joint involvement	15	0	15	0	15	3	12
Rheumatoid spondylitis with inactive peripheral rheumatoid arthritis	7	0	7	0	7	0	7
Psoriatic arthritis	9	0	9	0	9	1	8
Arthritis, type undetermined, possibly early rheumatoid arthritis	13	0	13	0	13	0	13
Other arthropathies (3)	30	0	30	0	30	2	28
Non-arthritic controls except infectious hepatitis (4)	28	0	28	0	28	Not done	
Infectious hepatitis	9	2	7	0	9	1	8

(1) A titer of 28 or higher with complete agglutination in first 2 tubes is considered positive.

(2) A differential titer of 16 or greater is considered positive.

(3) See Note No. 3—Table III.

(4) See Note No. 4—Table III.

modified test, and the resulting titer was positive in each case. No sera were encountered in which the Rose test was positive and our modified test negative. It is of interest that in the 4 cases of active peripheral rheumatoid arthritis which were negative by our test, both the Rose and the streptococcus agglutination tests were also negative.

**Discussion.** The agglutination of unsensitized sheep cells by human sera is due to a Forssman antibody (natural amboceptor).<sup>9</sup> This antibody may be present in varying con-

centrations or may occasionally be absent. It is not related to the hemagglutinin associated with active rheumatoid arthritis nor is it necessary for the reaction with sensitized cells. The factor in the sera of patients with rheumatoid arthritis responsible for the agglutination of sensitized sheep cells behaves like an antibody. This is suggested by its reaction in selective absorption studies and its identity as part of the beta-gamma globulin complex.<sup>1</sup> Our results indicate that this substance is different serologically from the natural amboceptor of human serum as well as from the heterophile antibody of infectious mononucleosis.<sup>7</sup> Evidence of its specificity is found

<sup>9</sup> Kabat, E. A., and Mayer, M. M., *Experimental Immuno-chemistry*, Charles C. Thomas, Springfield, Ill., 1948, p. 135.

TABLE V.  
Comparative Results in Those Cases of Active Rheumatoid Arthritis in Which the Rose Test Was Negative and the Modified Test Positive.

Diagnosis	Rose test			Modified test titer
	Normal RBC	Sensitized RBC	Differential titer	
Active peripheral rheumatoid arthritis (8 cases)	28	112	4	56
	7	56	8	28
	14	56	4	28
	28	224	8	224
	112	224	2	56
	14	112	8	224
	56	448	8	448
	14	112	8	448
Active peripheral rheumatoid arthritis with spondylitis (3 cases)	7	28	4	28
	112	224	2	112
	28	56	2	28

RBC refers to sheep red blood cells.

in the fact that it is present in a high percentage of proven cases of rheumatoid arthritis and cannot be demonstrated in any of the other arthritides or in a wide variety of unrelated medical controls. The occurrence of positive reactions in 2 cases of infectious hepatitis, both in low titer, may be due to the presence of altered globulins or unabsorbed antibodies associated with this disease. The concentration of rabbit anti-sheep amboceptor used in our test is based on its titer as determined by complement-fixation.<sup>1</sup> Our experience has shown that varying the concentration within critical limits will produce false reactions.<sup>7</sup> We believe the present method of determining the sensitizing unit of amboceptor is unsatisfactory. Standardization of the sensitizing unit should be based on the capacity of a particular pool of amboceptor to agglutinate a particular suspension of sheep cells rather than on its lytic capacity. Further studies on standardization along these lines are being pursued.

Natural amboceptor in human sera can contribute to the further sensitization of cells thereby increasing their agglutinability. When the rheumatoid arthritis hemagglutinin is present in high titer, the amount of naturally occurring amboceptor is not of practical significance because of dilution. For this reason there is no essential difference in the titer obtained by absorbed and unabsorbed sera against sensitized cells (Table I). However,

when the rheumatoid arthritis hemagglutinin is of relatively low titer, the normal amboceptor in the unabsorbed serum exerts an additive effect with the rabbit anti-sheep amboceptor. In this event, there is relatively little dilution and the titer may then be higher than that obtained by absorbed serum (Table V). Based upon our data, a titer of 1:28 with complete agglutination in the first two tubes can be considered positive. Thus far, we have found that the concentration of the rheumatoid arthritis factor does not necessarily correlate with clinical severity of the disease. On the other hand, in no case of inactive disease was a positive titer obtained. A serious limitation of the test as a diagnostic tool is that it appears to become positive only when x-rays begin to show the joint changes characteristic of rheumatoid arthritis. In the group classified as arthritis of unknown type in which the course was not inconsistent with rheumatoid arthritis but where no x-ray changes characteristic of the disease were present, the test was negative. It will be noted that the streptococcus agglutination test and the Rose test were also negative in all of these cases.

If the hemagglutinin associated with rheumatoid arthritis is indeed an antibody, investigation is required to determine whether this antibody is the result of immunization by an etiological agent or whether it is analogous to the non-specific antibody of the comple-

ment-fixation reaction which is almost always associated with syphilis. If it is a specific antibody, its absence in all cases of active rheumatoid spondylitis without peripheral joint involvement and in psoriatic arthritis is suggestive of different etiologies. The modified test, by eliminating the natural amboceptor and by more closely reflecting the titer of the rheumatoid arthritis factor, is more sensitive than the original hemagglutination test. A comparison of the 3 tests employed indicates that a significantly higher incidence of positive reactions can be obtained by this procedure.

*Summary.* 1. The hemagglutination test for rheumatoid arthritis is modified by the selective absorption of non-specific factors. The details of this modification are described.

2. Of 39 cases with active peripheral rheumatoid arthritis with x-ray evidence of joint changes, 35 were positive by this test.

3. All cases of inactive peripheral rheumatoid arthritis, rheumatoid spondylitis without

peripheral joint involvement and psoriatic arthritis tested were negative.

4. In presumptive cases of early rheumatoid arthritis without x-ray evidence of joint changes, the test was negative.

5. In 67 control cases, including a variety of non-rheumatoid arthropathies, the test was negative except in two cases of infectious hepatitis.

6. The modified test was positive in 90% of the proven cases of active peripheral rheumatoid arthritis tested; the Rose test in 61%; the streptococcus agglutination test in 58%. In all cases in which the modified test was negative, the Rose test and the streptococcus agglutination test were also negative.

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## Methyl Alcohol Purification of the Rabbit Papilloma Virus.\*† (17421)

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Rabbit papillomatosis is an endemic virus disease of cottontail rabbits readily transmissible to domestic rabbits. Serum from infected cottontail and domestic rabbits contains antibody which is capable of neutralizing the virus *in vitro*.<sup>1</sup> The virus can be obtained easily from cottontail rabbit papillomas, but ordinarily it is not recoverable from domestic rabbit growths.

The demonstration that extravasated fluid

from large confluent cottontail papillomatous growths was effective in neutralizing active virus led Kidd<sup>2</sup> to suggest that the neutralizing principle represented antibody derived through escape from altered capillaries. Kidd<sup>2</sup> found no evidence of such "masking" in naturally occurring papillomas of cottontail rabbits.

When Kidd<sup>2</sup> obtained no virus from the growths of 8 of 11 domestic rabbits and little from the other 3, and found that the sera of the 11 rabbits had little or no neutralizing capacity, he concluded that it was improbable that antibody was primarily responsible for the masking of the virus in the growths produced in domestic rabbits.

The method developed by Cox, van der Scheer, Aiston and Bohnel<sup>3</sup> for the purification and concentration of influenza and other

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<sup>1</sup> Shope, R. E., and Hurst, E. W., *J. Exp. Med.*, 1933, 58, 607.

<sup>2</sup> Kidd, J. G., *J. Exp. Med.*, 1939, 70, 583.

viruses by employing organic solvents at low temperatures to precipitate virus proteins led us to explore this technic in our efforts to characterize the agent responsible for the masking of the papilloma virus. Accordingly, experiments were designed to isolate the virus of infectious rabbit papillomatosis by precipitation (a) from crude tissue suspensions derived from naturally occurring cottontail papillomas, (b) from a suspension of virus artificially "masked" as the result of specific neutralization *in vitro*, and (c) from tissue suspensions derived from virus-induced domestic rabbit papillomas.

It is the purpose of this paper to report the results of our findings.

*Methyl Alcohol Purification of Cottontail Papilloma Tissue.* Four grams of cottontail papilloma tissue were ground with sand in a mortar to yield a 20% suspension in saline. After centrifugation in an angle centrifuge at 5,000 r.p.m. for thirty minutes at 4°C, the supernatant fluid was withdrawn. Ten ml of ether were added to 20 ml of the supernatant material. The mixture was shaken and centrifuged at 5,000 r.p.m. for 30 minutes at 4°C. After withdrawal of the lipid layer the remaining ether was removed *in vacuo*. The suspension was frozen rapidly at -20°C, thawed at room temperature, and centrifuged at 2,500 r.p.m. for 30 minutes at 4°C. The supernatant was drawn off, diluted to 200 ml with 0.1 M phosphate buffer, pH 7, cooled in a dry ice bath, and absolute methyl alcohol at -40°C was added drop by drop by means of a capillary pipette with constant stirring until the alcohol concentration reached 25%. The suspension was then kept in an ice bath for one hour at -2°C to allow time for the formation of the precipitate. The material was centrifuged at 5,000 r.p.m. for 30 minutes at 4°C. The alcohol was decanted and the precipitate washed with .02 M phosphate buffer, pH 5.1, to remove more non-viral protein. The addition of 4 ml of phosphate buffer, pH 7.0, to the resultant precipitate made available papilloma virus in a suspension of which 1 ml was equivalent to 1 gm

of tissue. After elution for 15 minutes, the suspension was frozen, thawed, and centrifuged at 2,500 r.p.m. for 30 minutes. The final water-clear supernatant fluid was kept in storage at -20°C until used.

*Tests for the Presence of Virus in Purified Material.* The purified viral suspension was diluted so as to have 8 successive two-fold dilutions ranging from 1-10 to 1-5120 to test for infectivity by transfer to domestic rabbits. Each dilution was inoculated in triplicate by the application of 0.2 ml by crosshatch scarification to previously shaved areas of skin on 3 rabbits. The results as observed 4 weeks later are recorded in Table I.

For control purposes to learn the infectious titer of the papillomatous tissue which was employed for methanol precipitative purification, infectivity tests in triplicate of the non-purified virus suspension in dilutions of from 1-10 to 1-6400 were carried out. Papillomatous growths were present 4 weeks later at all sites of inoculation.

It appears from these results that virus was lost in the process of purification, since the limiting dilution for infectivity of the purified virus was found to be 1-2560, whereas an end point was reached for the control material in a dilution of 1-6400.

These findings were established when the same technic for purification, concentration and biologic testing of 5 additional samples of cottontail papilloma tissue that had been collected between 1938 and 1944 yielded similar results. In each instance the purified virus suspension produced growths in dilutions through 1-2560 and the control virus suspensions yielded tumors in slightly higher dilutions, in most cases 1-6400.

*Methyl Alcohol Purification of Neutralized Papilloma Virus.* Cottontail papilloma tissue that had been stored in 50% glycerin at 4°C was ground to a fine paste in a mortar, and saline was added to make a 20% suspension. This was centrifuged at 2,000 r.p.m. for 20 minutes, and the supernatant material removed. Blood was drawn by cardiac puncture from a domestic rabbit which had borne several large papillomatous growths for about 3 months, and the serum was separated by centrifugation. Ten ml of serum was added to

<sup>3</sup> Cox, H. R., van der Scheer, J., Aiston, S., and Bohnel, E., *J. Immunol.*, 1947, **56**, 149.

TABLE I.  
Growths Produced with Purified Virus.

Rabbit	Reciprocal of dilution							
	10	40	160	320	640	1280	2560	5120
1	+	+	+	+	+	+	+	—
2	+	+	+	+	+	+	+	—
3	+	+	+	+	+	+	+	—

(+) Visible papillomatous growth.

(—) No visible growth.

an equal volume of the 20% viral suspension. The mixture was incubated at 37°C for one hour followed by refrigeration at 4°C overnight. For control purposes a 20% suspension of unneutralized virus was treated in the same manner. Following the incubation and refrigeration, 20 ml of the neutralized virus were subjected to the methanol purification procedure described previously. Four ml of phosphate buffer, pH 7.0, was added to the final precipitate. This suspension was allowed to elute for 15 minutes, frozen, thawed, and centrifuged at 2,500 r.p.m. for 30 minutes. The final supernatant represented the test inoculum. Three normal rabbits were prepared for inoculation by shaving and lightly scarifying 3 dermal areas. Each rabbit was then inoculated by cross-hatch scarification with the methyl alcohol purified neutralized virus in 0.2 ml amounts, and with control suspensions of neutralized virus and of crude virus. The control suspension had been carried through the same incubation and refrigeration procedures as the methyl alcohol purified material. After an incubation period of 5 weeks the rabbits were examined for signs of papillomatous growth. No growth appeared on any of the areas inoculated with the neutralized virus or with the methyl alcohol purified neutralized virus. Control inoculations of virus alone resulted in confluent papillomatosis.

The results indicated that methanol purification was ineffective for the separation of infective virus from the virus-antibody combination, since no visible papillomatous growths appeared in 6 rabbits after inoculation with the purified material. Three additional experiments failed to show that the virus could be extracted from the combination.

#### *Methyl Alcohol Purification of Domestic*

*Rabbit Papilloma Tissue.* Papilloma tissue was removed surgically from pure-bred Dutch domestic rabbits which had borne the growths for 6 weeks or more. The tissue was triturated with sand in saline to make a 20% suspension. This material was centrifuged at 5,000 r.p.m. for 30 minutes at 4°C. The supernatant material was drawn off and subjected to the same methyl alcohol purification procedure as was used on the cottontail papilloma tissue. A precipitate was obtained similar to that obtained with the purified cottontail papilloma tissue. Four areas on the ears and flanks of 4 normal pure-bred Dutch rabbits were inoculated by scarification with the purified material. The rabbits were examined for signs of growth at weekly intervals following the inoculation.

When no growths resulted on any of the rabbits inoculated over a 12-week period, it was concluded that the domestic papillomatous growths following treatment by the methanol technic for purification yielded insufficient active virus to cause visible papillomatous growths. Further attempts were made to extract virus from 4 samples of domestic papilloma tissue from rabbits which had borne the growths from 3 to 15 weeks. In no case was any demonstrable virus obtained.

*Summary.* The methanol precipitative technic of Cox, *et al.*, when applied to the purification and concentration of papilloma virus from naturally occurring cottontail rabbit papillomas, domestic rabbit papillomas and neutral virus-antibody mixtures readily yielded active papilloma virus from the cottontail papillomas but it did not yield virus from either domestic rabbit papillomas or from papilloma virus and its antibody in neutral mixture.



## Ineffectiveness of Racemic Glyceric Aldehyde in the Prevention of Experimental Tooth Decay. (17422)

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The striking ability of racemic glyceric aldehyde to inhibit acid production by the oral flora during the incubation of saliva was demonstrated in the experiments of Fosdick and Calandra.<sup>1</sup> This observation suggested the need to investigate the ability of dl-glyceric aldehyde to influence the initiation and development of carious lesions in experimental animals of known caries-susceptibility. In an earlier series of experiments conducted in this laboratory, additions of 0.5%, 1.0% and 2.0% crystalline racemic glyceric aldehyde to the caries-producing purified ration throughout the experimental period had no effect on the dental caries incidence of white rats and cotton rats.<sup>2</sup> In the above tests, the glyceraldehyde was present in the ration in the dimeric form and, presumably, passed through the oral cavity largely, if not entirely, as the dimer. Since the glyceraldehyde used in the experiments of Fosdick and Calandra was in solution, where some dissociation of the dimer to the monomer might have occurred, experiments with caries-susceptible animals were undertaken to determine if racemic glyceraldehyde given throughout the experimental period as the monomer influenced the dental caries experience.

**Experimental.** Six litters of white rats were used as the subjects of the first experiment. These animals were born to females which had been maintained throughout their reproductive lives on purified ration 100.<sup>3</sup> Previous litters from the same females had had a high caries-susceptibility. The 35 weanling rats from these litters were distributed into 3 groups. The 12 rats in the first group were fed ration 100 plus 10% Cellu flour and

allowed to drink tap water. The second group of 9 rats was offered the above ration supplemented with 0.5% crystalline dl-glyceric aldehyde and drank tap water. The third group of 14 rats was fed ration 100 plus 10% Cellu flour and was only allowed to drink tap water to which 0.2% dl-glyceric aldehyde had been added.

In the second experiment a litter of 6 weanling cotton rats was divided into 2 equal groups. The cotton rats in the first group served as controls and were offered ration 100 plus 10% Cellu flour and tap water; those in the second group were fed the same ration but 0.2% dl-glyceric aldehyde was incorporated into the drinking water.

In both experiments, food and drinking water were provided *ad libitum* and the rodents were housed in individual screen bottom cages. The dl-glyceric aldehyde solution was prepared sufficiently in advance of its use to permit dissociation of the dimer to the monomer. Tap water and the 0.2% solution of glyceraldehyde were offered to the animals in identical watering bottles. The 0.2% level of dl-glyceric aldehyde in the drinking water was selected on the basis of a comparison between the average food and water consumptions of these rodents. This value permitted the consumption of approximately the same amount of glyceraldehyde as the 0.5% supplement to the purified ration.

The first experiment was terminated at the end of 20 weeks and the second experiment at the end of 14 weeks. After fixation of the skulls in 95% ethanol, the molar teeth were examined to determine the number and size of the carious lesions.

**Results.** The average rates of growth of the white rats and cotton rats fed dl-glyceric aldehyde either in the ration or in the drinking water were the same as the average rates of growth of comparable control animals.

<sup>1</sup> Fosdick, L. S., and Calandra, J. C., *J. Dental Research*, 1947, **26**, 309.

<sup>2</sup> Shaw, J. H., *J. Dental Research*, 1948, **27**, 727; *ibid.*, to be published.

<sup>3</sup> Shaw, J. H., *J. Dental Research*, 1947, **26**, 47.

TABLE I.  
Caries Incidence of Rodents Fed Racemic Glyceric Aldehyde in the Ration or in the Drinking Water.

% glyceric aldehyde in supplement	No. of animals	No. of carious molars				No. of carious lesions				Extent of carious lesions			
		Avg	S.E.M.*	C.R.†		Avg	S.E.M.*	C.R.†		Avg	S.E.M.*	C.R.†	
0	12	5.7	0.8	0.3	Exp. 1. White rats ( <i>Mus norvegicus</i> )	7.6	1.1	0.4		18+	5+	0.4	
	9	6.0	0.7	0.1		8.4	1.5	0.5		21+	5+	0.3	
	14	5.6	0.5	0.5		6.9	1.0	0.8		16+	3+	0.8	
0.5 in ration					Exp. 2. Cotton rats ( <i>Sigmondon hispidus hispidus</i> )	25.7	3.5			77+	19+	0.3	
0.2 in drinking water	3	10.7	0.5	0		23.3	3.1	0.5		69+	13+		

\* Standard error of mean.

† Critical ratio—the ratio of the difference between 2 means to the standard error of the difference between the means. Wherever the critical ratio is less than 2.0, the difference between the means is considered to be statistically insignificant; when the critical ratio is between 2.0 and 2.9, the difference is of borderline significance; when the critical ratio is 3.0 or higher, the difference is highly significant. The criss-cross lines in the above table connect sets of data which are being compared for the critical ratio test of significance.

There was no evidence of a toxic effect of the glyceraldehyde additions.

The average caries experiences of the groups of white rats and cotton rats fed racemic glyceric aldehyde in the ration or in the drinking water, and of comparable control groups are presented in Table I. In the white rat, the consumption of a purified caries-producing ration supplemented with 0.5% of dl-glyceric aldehyde in the dimeric form did not influence the average number of carious molars, the average number of carious lesions, nor the average extent of tooth substance affected by the carious processes. Likewise, the consumption of water containing 0.2% dl-glyceric aldehyde as the monomer throughout the experimental period of maintenance on the purified ration did not alter the caries experience of both species.

**Discussion.** The results of the above experiments and of those reported previously<sup>2</sup> indicate the inability of racemic glyceric aldehyde, as the dimer or as the monomer, to alter the initiation and rate of development of carious lesions in experimental animals maintained under the conditions of these studies. These observations are in contrast to those which indicated the strong ability of glyceric aldehyde to inhibit acid production in incubated saliva. For a number of years, dental investigators in several laboratories have suggested that compounds which were able to in-

hibit acid production in incubated saliva or in incubated cultures of *Lactobacillus acidophilus* would have a similar inhibitory effect on the initiation and development of tooth decay in experimental animals and human beings. In view of the divergent results between *in vitro* studies and animal experiments with racemic glyceric aldehyde, the above generalization does not appear to be valid. Careful examination of suggestive *in vitro* findings by suitable *in vivo* procedures with caries-susceptible laboratory animals and later with human subjects is necessary to establish the exact value of any compound or treatment for caries prevention. The striking gross and histologic similarity between experimentally produced carious lesions in the Norway rat<sup>4</sup> and in the cotton rat<sup>5</sup> to the naturally occurring lesions in human teeth is indicative of the validity of these two species for the evaluation of *in vitro* inhibitors prior to clinical application.

**Summary.** The addition of 0.5% of dl-glyceric aldehyde to a caries-producing ration or of 0.2% dl-glyceric acid to the drinking water did not reduce the initiation nor the rate of development of carious lesions in caries-susceptible white rats and cotton rats.

<sup>4</sup> Sognnaes, R. F., *J. Nutrition*, 1948, **36**, 1.

<sup>5</sup> Shaw, J. H., *J. Nutrition*, 1949, **38**, 275.

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## Symbiotic Growth of Pleuropneumonia-like Organisms with Bacterial Colonies. (17423)

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A symbiotic relationship among bacteria was first described by Grassberger.<sup>1</sup> He observed that in the vicinity of colonies of various bacteria, particularly *Staphylococcus aureus*, *Hemophilus influenzae* grew as satellite

colonies on blood agar. It is now well known that the staphylococci synthesize necessary growth factors required by *Hemophilus influenzae* which are not contained in the medium.

A similar symbiotic relationship has been observed with the pleuro pneumonia-like organisms (P.PLO). In the course of our work

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<sup>1</sup> Grassberger, R., *Z. f. Hyg.*, 1897, **25**, 453.

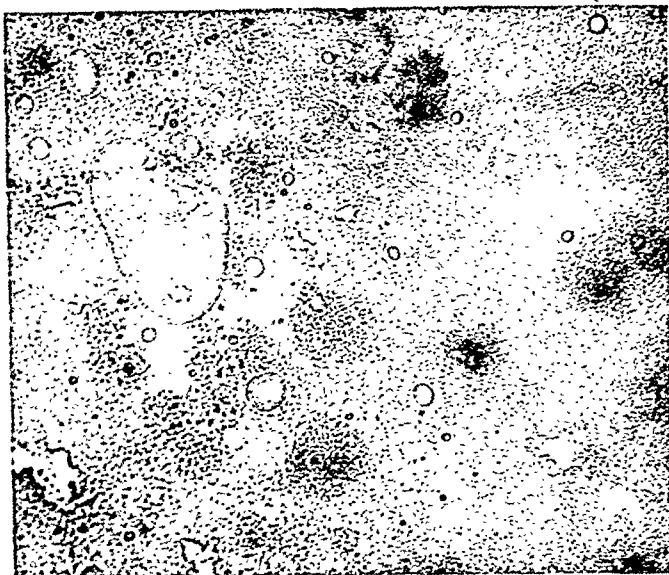


FIG. 1.

PPLO colonies, strain No. 17 from cervical secretion, growing under *Staphylococcus albus*. When the agar block containing the staphylococcal and PPLO colonies was stained by the method of Dienes, the entire bacterial growth took the blue dye. After a short time the staphylococcal colonies reduced the dye leaving the intensely stained PPLO colonies as distinct islands. 130 X.

with these organisms a gram-positive coccus occurred as a contaminant on a beef heart infusion agar plate (without ascitic fluid) which had been inoculated with the PPLO. The PPLO were noticed directly underneath the bacterial colonies. The gram-positive cocci were identified as *Staphylococcus albus*. An attempt was then made to determine whether this phenomenon would occur with other PPLO strains, many of which had been isolated from clinical sources. The PPLO were never observed to grow on heart infusion agar medium containing Bacto-beef heart 5.0%, Bacto-peptone 1.0%, NaCl 0.5%, and agar 1.5% unless ascitic fluid or blood serum was added. The entire areas of beef heart infusion agar plates without ascitic fluid were inoculated with the different strains of PPLO by streaking the surfaces with agar blocks containing the L type colonies. Inoculation of the staphylococci was accomplished by making one streak across each of the plates after the inoculation with PPLO. After five days incubation aerobically at 37°C nine of four-

teen strains from human sources and one strain from a rat grew underneath the staphylococcal colonies. In the isolated areas where there were no staphylococcal colonies the PPLO did not grow. This experiment was repeated using a strain of *Proteus vulgaris*. All 15 strains of the PPLO grew and, as was the case with the staphylococcus, only underneath the bacterial colonies. In neither case were any structures similar to the L type colonies observed in growths of the *S. albus* and *P. vulgaris* on the medium which was not inoculated with PPLO.

The colonies of PPLO were detected by direct microscopic examination of the plates at a magnification of approximately 100X. Their identity was verified by cutting out blocks of agar and staining the growths by the method of Dienes. Photographs of two stained preparations are reproduced as Fig. 1 and 2.

This report is made for possible usefulness to those interested in cultivating the PPLO from clinical materials or to those interested in the metabolism of this little-understood

There was no evidence of a toxic effect of the glyceraldehyde additions.

The average caries experiences of the groups of white rats and cotton rats fed racemic glyceric aldehyde in the ration or in the drinking water, and of comparable control groups are presented in Table I. In the white rat, the consumption of a purified caries-producing ration supplemented with 0.5% of dl-glyceric aldehyde in the dimeric form did not influence the average number of carious molars, the average number of carious lesions, nor the average extent of tooth substance affected by the carious processes. Likewise, the consumption of water containing 0.2% dl-glyceric aldehyde as the monomer throughout the experimental period of maintenance on the purified ration did not alter the caries experience of both species.

**Discussion.** The results of the above experiments and of those reported previously<sup>2</sup> indicate the inability of racemic glyceric aldehyde, as the dimer or as the monomer, to alter the initiation and rate of development of carious lesions in experimental animals maintained under the conditions of these studies. These observations are in contrast to those which indicated the strong ability of glyceric aldehyde to inhibit acid production in incubated saliva. For a number of years, dental investigators in several laboratories have suggested that compounds which were able to in-

hibit acid production in incubated saliva or in incubated cultures of *Lactobacillus acidophilus* would have a similar inhibitory effect on the initiation and development of tooth decay in experimental animals and human beings. In view of the divergent results between *in vitro* studies and animal experiments with racemic glyceric aldehyde, the above generalization does not appear to be valid. Careful examination of suggestive *in vitro* findings by suitable *in vivo* procedures with caries-susceptible laboratory animals and later with human subjects is necessary to establish the exact value of any compound or treatment for caries prevention. The striking gross and histologic similarity between experimentally produced carious lesions in the Norway rat<sup>4</sup> and in the cotton rat<sup>5</sup> to the naturally occurring lesions in human teeth is indicative of the validity of these two species for the evaluation of *in vitro* inhibitors prior to clinical application.

**Summary.** The addition of 0.5% of dl-glyceric aldehyde to a caries-producing ration or of 0.2% dl-glyceric acid to the drinking water did not reduce the initiation nor the rate of development of carious lesions in caries-susceptible white rats and cotton rats.

<sup>4</sup> Sognnaes, R. F., *J. Nutrition*, 1948, **30**, 1.

<sup>5</sup> Shaw, J. H., *J. Nutrition*, 1949, **38**, 275.

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## Symbiotic Growth of Pleuropneumonia-like Organisms with Bacterial Colonies. (17423)

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A symbiotic relationship among bacteria was first described by Grassberger.<sup>1</sup> He observed that in the vicinity of colonies of various bacteria, particularly *Staphylococcus aureus*, *Hemophilus influenzae* grew as satellite

colonies on blood agar. It is now well known that the staphylococci synthesize necessary growth factors required by *Hemophilus influenzae* which are not contained in the medium.

A similar symbiotic relationship has been observed with the pleuro pneumonia-like organisms (PPLO). In the course of our work

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<sup>1</sup> Grassberger, R., *Z. f. Hyg.*, 1897, **25**, 453.

studying trypsin-antitrypsin systems. The fibrinogenolytic method for measuring blood proteases and protease inhibitors, as first suggested by Ferguson,<sup>6</sup> or in somewhat modified form,<sup>7-9</sup> has proven especially useful. These procedures, however, leave something to be desired for routine tests on a large scale.

The need for a truly simple, quantitative technic for accurate titration of the changes in the protease inhibitor titers of body fluids associated with various physiologic and pathologic states, and particularly in connection with allergic phenomena, has been obvious for a long time. The concept that hypersensitivity reactions are caused primarily by toxic products formed, or released, when the natural proteases are suddenly activated following antigen-antibody union, was originally expressed many years ago, as was also the correlated idea that allergic reactions are controlled by the action of protease inhibitors.<sup>10-12</sup> Until recently very little precise data in direct support of this view has been published, however. The importance of supplying such data on an adequate scale led one of us some years ago to attempt development of a quantitative procedure for antitrypsin titration especially suitable for use by immunologists.<sup>13-15</sup> The method was based upon the fact that when properly prepared photographic

film is brought into contact with solutions of active trypsin the gelatin is digested, releasing the silver and clearing the film to a degree proportional to the activity of the trypsin. When digestion is complete the originally opaque, black film becomes completely transparent.

The present paper outlines the vastly improved technic for the performance of this film-gelatin-digestion method developed during the past two years.

**Reagents.** 1. *Stock buffer solution pH 7.5* is made by mixing 1.3 parts of M/20 sodium borate (19.1 grams  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$  per liter of distilled water) with 8.7 parts of M/5 boric acid-salt solution (12.4 grams  $\text{H}_3\text{BO}_3$  and 2.9 g NaCl per liter of distilled water). This reagent is used for all dilutions of trypsin and of sera or other fluids under test.

2. *M/5 sodium borate solution* (76.3 grams  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10 \text{H}_2\text{O}$  per liter of distilled water) is kept in a sealed bottle in a 37°C incubator.

3. *Stock crystalline trypsin solution (CT)* is prepared by dissolving 0.3 g of commercial crystalline trypsin<sup>†</sup> in 100 ml of a glycerin-buffer mixture consisting of equal volumes of stock buffer solution and C.P. glycerine; the pH is about 4.4. It is stored in a sterile, chemically clean, screw-top bottle at 4-10°C. Freshly-made solutions are allowed to stand undisturbed in the refrigerator 24 hours before use. (A precipitate appearing at this time is removed by filtering through paper.) The effect of the glycerine and the low pH is to stabilize the enzyme<sup>16</sup> so that the proteolytic activity of the solution remains unchanged for many months.

4. *Working trypsin solution (WT)* is made by mixing one part of stock trypsin solution with two parts of the M/5 sodium borate solution (Reagent 2). This reduces the concentration of trypsin to 0.1% (1 mg/ml) and the pH is brought to 6.8. It is practicable to make up this reagent in a volume of 100 ml or more. Freshly made solutions are allowed to stand undisturbed in the refrigerator for 24 hours before use. When stored in

<sup>4</sup> Glazko, A. S., *J. Clin. Invest.*, 1947, **26**, 364.

<sup>5</sup> Milstone, J. H., *Science*, 1947, **106**, 546.

<sup>6</sup> Ferguson, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1943, **52**, 243.

<sup>7</sup> Ferguson, J. H., Travis, B. L., and Gerheim, E. B., *Proc. Soc. Exp. Biol. and Med.*, 1947, **64**, 255.

<sup>8</sup> Christensen, L. R., *J. Clin. Invest.*, 1949, **28**, 163.

<sup>9</sup> Downie, G. R., and Clifton, E. E., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 138.

<sup>10</sup> Bronfenbrenner, J., *Proc. Soc. Exp. Biol. and Med.*, 1915-16, **13**, 42.

<sup>11</sup> Bronfenbrenner, J., and Schlesinger, M. J., *J. Immunol.*, 1918, **3**, 321.

<sup>12</sup> Rusznayak, S., *Deutsch. med. Wchnschr.*, 1912, **38**, 168.

<sup>13</sup> Burdon, K. L., and Lafferty, C., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 767.

<sup>14</sup> Burdon, K. L., *J. Bact.*, 1941, **41**, 62.

<sup>15</sup> Burdon, K. L., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 24.

<sup>†</sup> Worthington Biochemical Laboratory, Freehold, N. J., or Armour Laboratories, Chicago, Ill.

<sup>16</sup> Burdon, K. L., *Science*, 1941, **93**, 21.

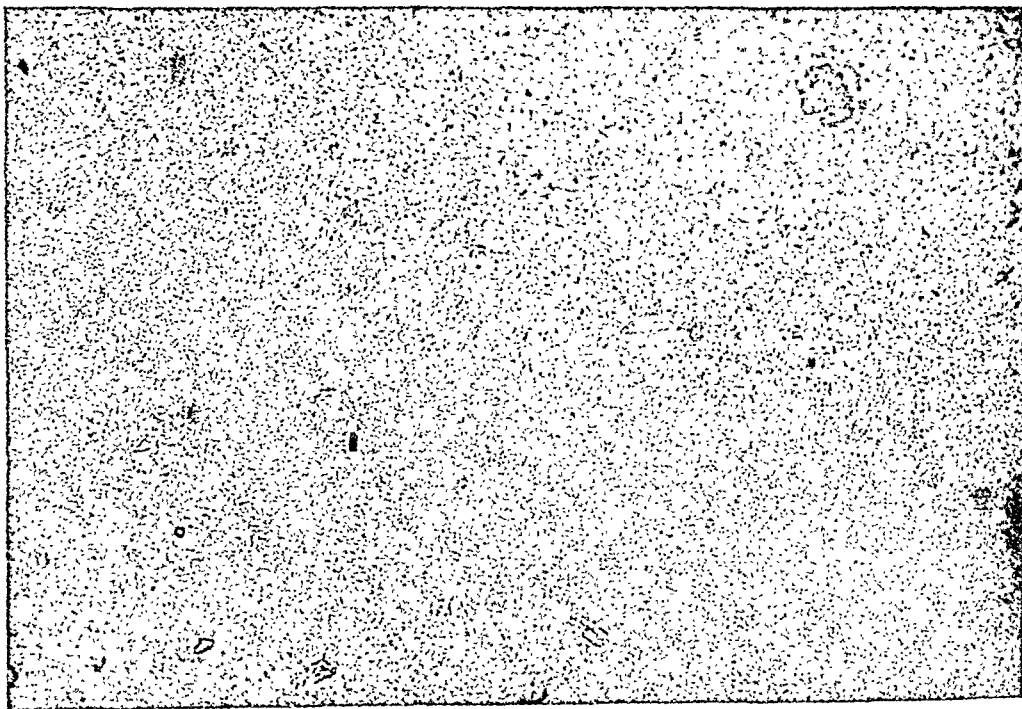


FIG. 2.

PPLO colonies, strain No. 36 from prostatic secretion, growing under *Proteus vulgaris*. When an agar block containing the growth of the two types of organisms was stained as described for Fig. 1 the PPLO colonies, with this strain, appeared as small darkly stained areas.

group of microorganisms.

**Summary.** Nine of 14 strains of PPLO of human origin and one strain of rat origin were observed to grow on beef heart infusion agar without ascitic fluid or serum only in sym-

biosis with *Staphylococcus albus* and all 15 strains grew in symbiosis with *Proteus vulgaris*.

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### A Simple, Quantitative Method for Titration of Trypsin and Trypsin-Inhibitors.\* (17424)

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In recent years the interest of an increasing number of investigators in the mechanism of fibrinolysis<sup>1</sup> and related phenomena, and especially in the role played by the activators

and inhibitors of natural plasma proteases in the complicated process of blood coagulation,<sup>2-5</sup> has led to improved procedures for

<sup>1</sup> Christensen, L. R., *J. Gen. Physiol.*, 1945, **28**, 363.

<sup>2</sup> Ferguson, J. H., *Science*, 1943, **97**, 139.

<sup>3</sup> Tagnon, H. J., *J. Lab. and Clin. Med.*, 1942-43, **27**, 1119.

\* This investigation was supported in part by a grant from the Division of Research Grants and Fellowships of the National Institutes of Health, United States Public Health Service.

other fluid under test.

All dilutions are made with the stock buffer solution in chemically clean glassware, using plugged, 1 ml serological pipettes. The trypsin control dilutions have a pH of 7.5 and succeeding tubes in any part of the test series differ in hydrogen ion concentration by only about 0.01 pH.

These dilutions gradually lose activity at room temperatures, especially at temperatures over 85° F., but the same set of solutions may be used throughout a working day if the tubes are kept in a pan of ice water, or if they are placed in the refrigerator except when actually being used.<sup>§</sup>

3. *Preliminary adjustment of the incubation time.* A new working trypsin solution is first titrated in dilutions 1 to 7 under the standardized conditions of the test proper, as outlined below, except that the digestion incubation time in the 40°C water bath is varied in successive runs until the time required to give the end-point regularly with dilution No. 5 (Table I) is determined. The tube made up with dilution No. 5, plus an equal volume of stock buffer, will thus have, as intended, just one unit of tryptic activity, i.e. it will contain the amount of WT just sufficient to digest all the gelatin (clear the film completely) in a certain time at 40°C. It will be seen (Table I) that the final concentration of crystalline trypsin in this end-point tube is 0.05 mg./ml. The time required with the present lot of film used by the writers is 6½ minutes.

First tests are commonly run at times differing by half-minute time intervals, then by quarter-minutes and finally by one-eighth minutes. The incubation time, once chosen on the basis of these tests, is kept constant throughout all future tests with that lot of working trypsin solution, and indeed, rarely requires any adjustment so long as the same

stock trypsin solution is used. Thus, strictly comparable titrations can be obtained in separate runs carried out weeks or months apart.

4. *The test proper.* a. Ordinary serological tubes (12 x 75 mm) are arranged in a water bath rack. Using a separate, plugged 1.0 ml pipette for each dilution, 0.4 ml of dilutions 3, 4, and 5 of the working trypsin solution is introduced into a series of 3 tubes that are to serve as trypsin controls, and 0.4 ml of other appropriate dilutions is placed in a further series of 6 or 8 additional tubes. Now, 0.4 ml of the stock buffer solution is added to each of the control tubes, and 0.4 ml of the diluted serum, or other fluid under test, to each of the remaining tubes. The rack is now shaken, as in a Kahn test, for one-half minute, then placed in a 40°C water bath<sup>¶</sup> with a timer set for 10 minutes.

b. The filmstrips are now obtained from their jar in the refrigerator and arranged in parallel rows on some convenient holder (such as a small slide-box lid) so that they can be more readily picked up one by one with forceps.

c. At the end of the warming period the interval timer is carefully reset for the test incubation time, e.g. 6½ minutes. The timer is started and the introduction of the filmstrips into the test tubes (still in the water bath) is begun immediately. The strips are added one by one in a definite order, and following a rhythmic time pattern which must be perfected by practice.

d. The fixing solution and equipment are now obtained from the refrigerator. Some of the solution is poured into a 600 ml beaker and heavy-walled capillary pipettes with large rubber bulbs attached are filled with it.

e. When the timer rings the rack is removed immediately from the water bath, and then, without delay, the cold fixing solution is added to each tube in the same order, and following the same time pattern in which the filmstrips were originally introduced. The fixing solu-

<sup>§</sup> The stability of the reagents and the effects of various other factors upon the validity of the tests have been experimentally studied, and findings will be reported in a separate communication.

<sup>¶</sup> The fact that the powdered commercial crystalline trypsin contains approximately 50% MgSO<sub>4</sub> is disregarded.

<sup>¶</sup> Best results have been obtained by the use of an Army-type electric water bath (Chicago Surgical and Electrical Co.) with separate gable cover over each rack.



TABLE I.  
Scheme for Dilution of Working Trypsin Solution.

Dilution No.	Working trypsin, ml*	Buffer, ml*	Trypsin conc., mg/ml		Tryptic units (TU)
			In dilution	Final†	
1	.4	7.6	.05	.025	.5
2	.5	7.5	.063	.031	.62
3	.6	7.4	.075	.037	.75
4	.7	7.3	.087	.044	.87
5	.8	7.2	.1	.05	1.0
6	.9	7.1	.113	.056	1.12
7	1.0	7.0	.125	.062	1.25
8	1.1	6.9	.138	.069	1.37
9	1.2	6.8	.15	.075	1.5
10	1.3	6.7	.163	.081	1.62
11	1.4	6.6	.175	.087	1.75
12	1.5	6.5	.188	.094	1.87
13	1.6	6.4	.2	.1	2.0
14	1.7	6.3	.213	.106	2.12
15	1.8	6.2	.225	.112	2.25
16	1.9	6.1	.238	.119	2.37
17	2.0	6.0	.25	.125	2.5
18	2.1	5.9	.263	.131	2.62
19	2.2	5.8	.275	.137	2.75
20	2.3	5.7	.288	.144	2.87
21	2.4	5.6	.3	.15	3.0

\* The amounts required to make up a total volume of 8.0 ml are shown because this is often a convenient quantity for routine tests.

† After mixture with equal volume of buffer, or diluted specimen, as in the test proper.

sterile, chemically clean screw-top bottles at 4-10°C the tryptic activity remains constant for at least 6 months.

5. *The fixing solution* consists of 95% ethyl alcohol to which 10% by volume of 28% glacial acetic acid is added.

6. *The filmstrips* are cut from the special film prepared to our specifications by the Eastman Kodak Company.‡ The film is a duplitized, 35 mm ciné-film, flashed on both sides to a density of 1.0, so that it has a total density of about 2.0. Both the density and the hardness of the emulsion are uniform throughout. The reels of stock film are kept at all times in tightly sealed cans in the refrigerator. A supply of strips is made, when needed, by cutting the film crosswise into pieces  $\frac{1}{4}$  inch wide with a small paper cutter. The width of the strips need not be exact. About  $\frac{1}{4}$  inch from the end each strip is bent

upward at nearly a right angle to make it easier to grasp with forceps. In handling the film it is important to avoid finger marks, and exposure to heat or dust. The newly-cut strips are transferred at once to clean, screw-top jars or tubes, and thus kept carefully sealed in the refrigerator until actually used in a test.

1. *Trypsin Dilution Method.* 1. *Initial dilution of the serum or other fluid to be tested.* The majority of specimens of normal human, rabbit, or guinea pig sera will give end-points in a convenient range when they are diluted originally with stock buffer 1:50. For some high-titer sera, or other fluids, it may be found desirable to use a different initial dilution (e.g. 1:100). In any series of comparative tests all specimens should be titrated at the same initial dilution.

2. *Dilutions of the working trypsin solution.* For the *trypsin control* tubes in routine tests it is necessary to prepare only dilutions 3, 4, and 5 (Table I). For the *test mixtures* a series of six or eight additional dilutions are usually made through a range likely to give end-points in the presence of the serum or

‡ We wish to express our sincere appreciation to Dr. Burt H. Carroll of the Research Laboratories, and Mr. W. F. Swann of the Industrial Photographic Sales Division for their generous cooperation in the development and production of this essential material.

Card No. <u>610</u>		Date <u>1/15-2/9/49</u>		<b>CONTROL TRYPSIN TITRATION</b>						References									
Trypsin, Lot. <u>X-CRYSTALLINE</u>		Method <u>TRYPSIN DILUTION</u>		DIL. No.	3	5	7		BUFFER		E Titer	Cards							
Film <u>D-16(3)</u>		Temp. <u>40°C.</u>		T.U.	0.75	1.0	1.25					Records							
Expt. <u>G.P. VIII</u>		Time <u>4 3/4 minutes</u>		WCT 131	●				●		1.0	Entry, h. 30, 31							
				WCT 131	●				●		1.0								
<b>ANTITRYPSIN TITRATION</b>																			
DIL. No.	8	9	10	11	12	13	14	15	16	17	18	19	20	21	TITER	MINUS TITER	X DIL.	A-T INDEX	
T.U.	1.375	1.5	1.625	1.75	1.875	2.0	2.125	2.25	2.375	2.5	2.625	2.75	2.875	3.0					
1	●	●	●	○											2.0	1.0	100	100	
2	●	●	●	●	●	●	●	●	●	●	●	○			3.0	2.0	100	200	
3																			
4																			
Specimens	No. 1	<u>GUINEA PIG #27 - NORMAL SERUM (BEFORE INJECTION)</u>																	
	No. 2	<u>GUINEA PIG #27 - SERUM 23 DAYS AFTER INJECTION WITH H-37 Rv</u>																	
	No. 3																		
	No. 4																		

FIG. 2.

ing figures for undiluted specimens H-105, G.P. No. 27 normal, and tuberculous (Fig. 2) are 8.75, 5.0, and 10 mg/ml respectively.

Again, the antitryptic effect may be compared with that exhibited by a chemically purified material, such as commercial crystalline soybean trypsin inhibitor (CSBI).<sup>†</sup> We have found that one TU (*i.e.* a working trypsin solution which in final dilution contains 0.05 mg/ml CT) is *completely* inhibited by only 0.0167 mg/ml CSBI, or a proportion of inhibitor to trypsin of 1:3, and that *partial* inhibition continues so long as the final concentration of CSBI is not less than one part to 5.6 parts of trypsin. In the presence of 6 parts of trypsin the film is completely digested. Each milliliter of *undiluted* specimen H-93 (Fig. 1) may thus be calculated to have shown an inhibiting power equivalent to that of 0.625 mg/ml of CSBI,—0.0875 mg/ml (the amount of trypsin present in the end-point tube) minus 0.05, divided by 6 = 0.00625 mg/ml × the final serum dilution (1:100) = 0.625 mg/ml. For H-105, G.P. No. 27 normal, and tuberculous, the figures are 1.46, 0.833 and 1.66 mg/ml respectively.

Finally, advantage may be taken of the fact that the relative amount of clearing of the filmstrips in the several tubes just pre-

ceding the end-point tube is as reproducible as the end-point itself, and these strips may be read in terms of *percentage of digestion*, thus furnishing in some instances a distinction between two specimens, both of which have shown the same end-points. In other words, antitryptic indexes based on end-points do not always express all the degrees of trypsinase-inhibiting activity possessed by different sera, even when the differences between the succeeding trypsin dilutions used is as small as is practicable, *i.e.* only 0.125 tryptic units, as in Table I. The estimate of the *percentage digestion* is made by close comparison under uniform illumination of the film discs in question with a standard set of discs showing 20 graduations from zero to 100% digestion. These readings are valuable in reinforcing the information obtained by comparison of end-points, and could be utilized for further statistical analysis of the findings.

**II. Specimen Dilution Procedure.** The purpose in this form of the test is to determine the highest dilution of the serum or other fluid under test that will inhibit in any degree the complete clearing of the film in the presence of one TU of trypsin at the standard time and temperature.

**1. Preliminary steps.** A sufficient volume

Card No. <u>602</u>		Date <u>1/17/49</u>		CONTROL TRYPSIN TITRATION										References									
Trypsin, Lot <u>X-CRYSTALLINE</u>		Method <u>TRYPSIN DILUTION</u>		DIL. No. <u>3</u>		<u>5</u>		<u>7</u>				BUFFER		C TITER		Cards							
Film <u>D-16 (3)</u>		Temp. <u>40°C.</u>		T.U. <u>0.75</u>		<u>1.0</u>		<u>1.25</u>						<u>10</u>		Records							
Spec. <u>HUMAN - TB.</u>		Time <u>4 3/4 minutes</u>		WCT 131 <u>2.1</u>										<u>10</u>		Entry, h. 44							
				WCT 131 <u>6.0</u>												HG-RB, h. 206							
ANTITRYPSIN TITRATION																							
Dil. No.		<u>6</u>	<u>7</u>	<u>8</u>	<u>9</u>	<u>10</u>	<u>11</u>	<u>12</u>	<u>13</u>	<u>14</u>	<u>15</u>	<u>16</u>	<u>17</u>	<u>18</u>	<u>19</u>	Titer		MUTUS C TITER		X DIL. =		A.T. INDEX	
T.U.		<u>1.125</u>	<u>1.25</u>	<u>1.375</u>	<u>1.5</u>	<u>1.625</u>	<u>1.75</u>	<u>1.875</u>	<u>2.0</u>	<u>2.125</u>	<u>2.25</u>	<u>2.375</u>	<u>2.5</u>	<u>2.625</u>	<u>2.75</u>								
1		●	●	●	●	○	○	○	○	○	○	○	○	○	○	<u>1.75</u>		<u>.75</u>		<u>100</u>		<u>75.0</u>	
2		●	●	●	●	●	●	●	●	●	●	●	●	●	○	<u>2.75</u>		<u>1.75</u>		<u>100</u>		<u>175.0</u>	
3																							
4																							
Specimens		No. 1 <u>NORMAL HUMAN SERUM, ♂ H 93</u>																					
		No. 2 <u>HUMAN SERUM, FAR ADVANCED PULMONARY TUBERCULOSIS, ♂ H 105</u>																					
		No. 3																					
		No. 4																					

FIG. 1.

tion is added with sufficient force to cause the strips to float upward somewhat, thus bringing the fixative into contact with the film throughout its length. The rack is now placed in the refrigerator for 5 minutes to assure the full hardening of any of the gelatin emulsion remaining undigested.

f. The filmstrips are now removed from the tubes with forceps, rinsed briefly in fresh, cold fixing solution, and touched, at the edges only, to blotting paper. They are then inverted and set upright in a bed of plasticine so that they will drain and dry uniformly.

5. *Reading of the test; preparation of the permanent record card.* By simple inspection of the dried strips when held to the light, the end-points (the first completely cleared films) in the trypsin control, and in the test series, are determined and recorded. From each strip a disc 5 mm in diameter is then cut from the digested portion with an ordinary paper punch. These discs are mounted, with the aid of Scotch tape, upon a white card which bears the desired descriptive data. Thus a permanent record card for filing may be made of every test. (Fig. 1, 2).

6. *Expression of the results.* The titer of proteolytic activity may be expressed most conveniently in terms of tryptic units (TU).

By preliminary adjustment of the incubation time, as described above, the titer in the trypsin control tubes (in all properly performed tests) is always 1.0 TU. The difference between this titer and that obtained in the test series of tubes in the presence of a specimen of serum or other inhibiting material represents the antitryptic power of that sample. A final figure to represent the *antitryptic index* is obtained by multiplying this difference by the final dilution (*i.e.* double the initial dilution) of the specimen. For example, in the presence of specimen H-93 (Fig. 1) the titer is 1.75 TU, a difference of 0.75 over the control titer of 1.0 TU. This difference, times the final dilution, gives the antitryptic index:  $0.75 \times 100 = 75.0$ .

The antiprotease action may also be expressed more directly in terms of the amount of trypsin inhibited. Thus specimen H-93, in final dilution 1:100. (Fig. 1) may be said to have prevented the activity of 0.0375 mg/ml of trypsin, the difference between the 0.0875 mg/ml in the first tube showing 100% digestion (No. 11) and the 0.05 mg/ml present in the control end-point tube (No. 5). Each milliliter of *undiluted* H-93 has the apparent capacity to inhibit the action of 3.75 mg/ml crystalline trypsin. The correspond-

moreover, is greatest when the inhibitor content of the specimen is especially high. Evidently the impurities in crude trypsin solutions prevent effective contact or combination of a considerable proportion of the antitryptic substances with the active trypsin molecules.

Despite these limitations, antitrypsin titrations with crude trypsin extracts have revealed data of apparent significance in support of the autodigestion theory of allergy.<sup>15,17</sup> For critical studies, however, only tests with crystalline trypsin can now be recommended.

*Comment.* It is important to realize that all of the procedures outlined above require meticulous attention to detail, and can only be carried out successfully by a conscientious technician, since the slightest deviation from the required technic shows up inexorably. Nevertheless, the tests are readily adaptable to routine use and an average of 20 separate specimens a day may be titrated by a single, practiced worker.

The method makes it feasible to add to the data ordinarily obtained in experimental and clinical studies of allergy precise information on the protease-inhibiting power of the sera of patients and experimental animals, or of the reagents used. The titrations should aid materially in appraising the significance of the

previously-neglected antitryptic effect *in vivo* or *in vitro* of the materials and manipulations employed in any experiment.

The basic film-gelatin-digestion technic should prove readily adaptable also for the titration of proteases other than trypsin, for experiments on the kinetics of these enzyme reactions and for the study of important physiologic and pathologic phenomena, other than hypersensitivity, in which natural proteases, their activators and inhibitors, appear to play so vital a part.

*Summary.* A technically simple, rapid method for the quantitative titration of trypsin or similar proteolytic enzymes, and of protease inhibitors, suitable for critical tests on a large scale is described. The procedure utilizes technics familiar to serologists, rather than to skilled biochemists only, and gives definitive, reproducible results that can be expressed in simple figures. A feature is the provision for the making of a permanent record of each titration. Since the reagents are stable, and readily standardized, comparable findings can be obtained in separate experiments conducted in the same or in different laboratories at any time.

The assistance of Mrs. David A. Cook with records and statistics is gratefully acknowledged.

<sup>17</sup> Burdon, K. L., and Mudd, R. P., *Proc. Soc. Exp. Biol. and Med.*, 1949, in press.

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## Quantitative Paper Chromatography: A Simplified Procedure. (17425)

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The now classical paper chromatographic method of Consden, Gordon and Martin<sup>1</sup> has been applied to the separation of many types of organic and inorganic substances. Although the method as originally described was only qualitative or roughly quantitative ( $\pm 50\%$ ), a number of quantitative adaptations have

been published. These adaptations may be classified into several general categories: (A) the paper chromatogram is cut so that each substance separated may be extracted with the appropriate solvent and then the concentration of the material in the extract is determined by conventional colorimetry or other means (*cf.*<sup>2</sup>); (B) the materials on the chromatogram are revealed by their own color.

<sup>1</sup> Consden, R., Gordon, A. H., and Martin, A. J. P., *Biochem. J.*, 1944, 38, 224.

of the working trypsin solution is made up in dilution No. 5 (Table I) to supply the 0.4 ml required for a test with each of 8 or 10 specimen dilutions, plus a control. Small amounts of dilutions 3, 4, and 5 are also made up separately to be run as independent trypsin controls. The specimen is diluted with stock buffer by the two-fold dilution procedure generally used in agglutination titrations, or by some other convenient scheme. The great majority of serum specimens from human beings, guinea pigs, or rabbits will give titers within the range 1:64 to 1:2500.

2. *The test proper.* To 0.4 ml of each of the specimen dilutions is added 0.4 ml of WT, dilution No. 5. Control tubes containing 0.4 ml of this identical trypsin solution + 0.4 ml of buffer, as well as the separately-made trypsin controls, are set up. The remaining steps of the test are now carried out as in the Trypsin Dilution Method outlined above.

3. *Expression of the results.* The titer of antitryptic activity here exhibited is ordinarily stated in terms of the highest final dilution of the specimen that inhibits in any degree the clearing of the film. The controls must show that the trypsin solution used in all the test mixtures contained, as intended, one TU. The results may also be stated in terms of the amount of trypsin inhibited, or they may be interpreted in comparison with the action of known amounts of crystalline soy bean inhibitor. A reading of the percentage of digestion at the titer dilution may also be made, differing degrees of inhibiting action may thus be revealed in different sera having the same dilution titer.

- *Use of crude trypsin.* The film-gelatin digestion method was originally developed before crystalline trypsin preparations became available commercially, and the procedures outlined above may still be used without essential change for tests with extracts of powdered crude tryptsins. The stock trypsin solutions are made by weighing out 6 g of Fairchild's trypsin,\*\* or 3 g of other commercial preparations†† and placing the pow-

der in a mortar. One hundred milliliters of glycerine-buffer solution (equal parts of C.P. glycerine and stock buffer) are added gradually, stirring in a small amount at first to make a paste. The mixture is transferred to a stoppered flask and allowed to stand at room temperature for about 2 hours, with occasional stirring, then overnight in the refrigerator. A crystal clear extract (pH about 5.8) is finally obtained by filtering through S and S No. 588 paper, or preferably through a Seitz filter after adding one percent of filter-aid.‡‡ These glycerin-buffer extracts are stable over long periods of time<sup>10</sup> when stored in a chemically-clean, screw-top bottle in the refrigerator.

*Comparative activity of crude and crystalline trypsin solutions; differences in reaction with trypsin inhibitors.* Crystalline trypsin solutions were found to have almost exactly the same activity in the filmstrip tests as the extracts obtained from 10 times the weight of the currently available crude powdered tryptsins. Hence, with the crude trypsin extracts made as described above end-points are obtained in a similar range of dilutions as with crystalline trypsin, and the same scheme of tryptic units may be used to express the titers obtained.

However, the amount of trypsin inhibitor revealed in serum and other materials is much greater in tests with crystalline trypsin than in parallel titrations with the less pure enzyme preparations. Thus, the antitryptic indexes of various sera tested with the newer powdered tryptsins were found to be about twice those obtained in titrations with the older, relatively impure, Fairchild's product. Crystalline trypsin again regularly reveals more than twice as much inhibitor as is shown in comparable tests with any of the currently available crude tryptsins. Thus, the average antitryptic index of 23 human sera, (initial dilution 1:50), tested in parallel, was 51.6 with one of the powdered tryptsins and 114.1 with crystalline trypsin. The difference,

†† ("Trypsin 1:300"), Nutritional Biochemicals Corporation, Cleveland, Ohio, or "Pancreatic Trypsin," Bios Laboratories, N. Y.

‡‡ Hyflo Super-Cel, Fisher Scientific Co., Pittsburgh, Pa.

\*\* Fairchild Bros. and Foster, N. Y. ("Trypsin 1:70"). This material is no longer being manufactured.

TABLE I.  
Standard Curves for Tyrosine and Histidine.

	mg	A	D	A × D	Factor
Tyrosine	.0090	18.5	.22	4.07	.0022
	.0090	26.5	.14	3.71	.0024
	.0090	16.5	.24	3.96	.0023
	.0180	27.0	.28	7.56	.0024
	.0180	40.5	.20	8.10	.0022
	.0180	24.5	.30	7.35	.0024
	.0180	37.5	.22	8.25	.0022
	.0180	29.5	.25	7.38	.0024
	.0270	53.2	.20	10.64	.0025
	.0270	50.0	.24	12.00	.0023
	.0270	28.0	.38	10.64	.0025
					.0024
Histidine	.0078	12.5	.32	4.00	.0020
	.0078	12.0	.34	4.08	.0019
	.0156	18.5	.43	7.96	.0020
	.0156	16.5	.41	6.77	.0023
	.0156	20.5	.40	8.20	.0019
	.0156	22.0	.38	8.36	.0019
	.0156	16.5	.43	7.10	.0022
	.0156	26.5	.26	6.89	.0023
	.0234	30.0	.35	10.50	.0022
	.0234	21.3	.56	11.93	.0020
					.0021

A—Area. D—Density. Factor—mg/A × D.

TABLE II.  
Tyrosine and Histidine in Proteins.  
(Calculated to 16.0 g of nitrogen).

Protein	mg used	Tyrosine, g	Histidine, g
Hemoglobin	.04-.32	3.0	8.2
Casein	.10-.60	5.9	2.9
Zein	.15-.90	5.1	1.8

dine and tyrosine ratios of which differ widely. These results are summarized in Table II. The values obtained agree with the best in the literature (*cf.*<sup>6</sup>).

2.) Estimation of Amino Acids on Two-Dimensional Paper Chromatograms. During investigations on one-dimensional chromatograms,<sup>4</sup> it was observed that the areas obtained from curves made from the maximum and minimum densities were almost as satisfactory as those prepared from scanning the entire curve. This led to an investigation of the relationship between the maximum color density of the spots on a two-dimensional chromatogram and the concentrations. The following procedure can be used to estimate 14 amino acids on approximately 0.3 mg of hydrolysate N of a protein with the usual

type of amino acid pattern, with an average error of less than 10%. Only 5 hours total working time are required. The elimination of the need for determining the area of the spot<sup>4</sup> is an obvious advantage.

*Experimental.* The protein or peptide is hydrolyzed with HCl or H<sub>2</sub>SO<sub>4</sub> and the excess mineral acid is removed in the usual fashion. The solution of amino acids is then diluted so that it contains approximately 1.5 to 10.0 millimols of each amino acid per ml. Isopropanol (to 10%) is used as the preservative. 0.005 or 0.01 ml aliquots of the hydrolysate are applied to the paper and the amino acids are then separated by two-dimensional chromatography on S. & S. No. 596, Whatman No. 4, or other suitable paper. Water-saturated phenol (in an atmosphere of 0.3% of NH<sub>3</sub>, moistened NaCN and coal gas) is the first solvent. The chromatograms are then dried in the hood with a blast of warm air until most of the solvent has been removed. Then the sheets are dried before an electric fan over night. 2.5 cm of the leading edge of the phenol run are cut off the paper in order to eliminate the discolored front. The chrom-

by reaction with specific reagents to produce a colored compound, by their microbiological effects when the paper is placed on a suitable test medium, or by radioactivity. In such instances, it has been found by Fisher, Parsons and Morrison<sup>3</sup> that the area on the paper occupied by each substance is proportional to the logarithm of its concentration. This procedure is very satisfactory when the boundaries of the spots are sharply delineated, but cannot be applied with accuracy unless this is so or when the substances are not distinctly separated from each other. (C) In order to overcome these disadvantages, a method has been described<sup>4,5</sup> in which the color density is determined continuously or in overlapping increments with an electronic densitometer along the entire length of a one dimensional chromatogram strip. The color densities, so obtained, are plotted on graph paper, a series of peaks are obtained, and the concentration of each substance or substances is calculated from the area under each density peak. This procedure is very satisfactory for the determination of substances which are well or partly separated from each other, but is exceedingly tedious unless automatic equipment is available. Furthermore, as there is no known single solvent which permits satisfactory separation of all or almost all the amino acids present in a protein hydrolysate, preliminary separation by adsorption, ion exchange or paper chromatography must be resorted to in many instances.<sup>4,6</sup> (D) One of the advantages of the paper chromatogram technic of Consden, Gordon and Martin<sup>1</sup> over the older capillary analysis method of Schoenbein and Goppelsroeder (cf.<sup>2</sup>) is the two-dimensional chromatogram. The method often gives even greater

resolution than can be obtained by preliminary separation by adsorption, ion exchange or large scale paper chromatography followed by one-dimensional strip chromatography and is much easier to conduct. It has been reported<sup>4</sup> that the concentration of a colored substance on a two-dimensional paper chromatogram can be estimated from the product of the greatest color density times the area of the spot.

The data to be given in this paper show that the concentration of colored substances separated by paper chromatography may be estimated directly on the chromatograms, with reasonable accuracy, either from the product of the area times the color density or more simply from the color density alone. The examples given are for the estimation of amino acids. However, equal success has been achieved with non-volatile amines and it appears that the same principle would apply to other colored substances on paper chromatograms.

1.) Determination of Histidine and Tyrosine in Protein Hydrolysates (cf.<sup>7</sup>) *Experimental.* Histidine and tyrosine are separated by paper chromatography employing S. & S. 598 paper with a solvent mixture consisting of N-butanol 100 parts, glacial acetic acid 10 parts, saturated with water. The chromatograms are run in air tight glass chambers (cf.<sup>8</sup>) for 3 hours. The paper is then removed, dried in air, and sprayed with freshly diazotized sulfanilamide dissolved in N-butanol. The chromatogram is again dried in air for exactly 5 minutes and then it is sprayed with saturated  $\text{Na}_2\text{CO}_3$  solution. The paper is dried in air and the quantities of histidine and tyrosine are determined from the product of the area of the spot times its maximum color density.<sup>4</sup>

*Results.* A few typical results on standard solutions are given in Table I. In order to ascertain the value of this procedure on protein hydrolysates, histidine and tyrosine analyses were carried out on 3 proteins, the histi-

<sup>2</sup> Schoenbein, C. F., *Verh. d. Naturforsch. Ges. Basel*, 1861, **3**, 249; Rheinboldt, H. in Houben, J. *Die Methoden der Organischen Chemie*, G. Thieme, Leipzig, 1925, **1**, 291.

<sup>3</sup> Fisher, R. B., Parsons, D. S., and Morrison, G. A., *Nature*, 1948, **161**, 764.

<sup>4</sup> Block, R. J., *Science*, 1948, **108**, 608.

<sup>5</sup> Bull, H. B., Hahn, J. W., and Baptist, V. H., *J. Am. Chem. Soc.*, 1949, **71**, 550.

<sup>6</sup> Block, R. J., and Bolling, D., *The Amino Acid Composition of Proteins and Foods*, C. C. Thomas, Springfield, Ill., 2nd ed., 1950.

<sup>7</sup> Bolling, D., Sober, A. H., and Block, R. J., *Fed. Proc.*, 1949, **8**, 185.

<sup>8</sup> Nachod, F. C., *Ion Exchange: Theory and Application*, Academic Press, New York, 1949, p. 308.

acids are absent or present in comparatively small amounts in the hydrolysate under analysis, the addition of known quantities of this amino acid to the hydrolysate will serve as an internal standard.

When 25 replicate chromatograms are run, the average per cent error for a mixture of 14 amino acids in proportions simulating  $\beta$ -lactoglobulin, was -2% in one series and -4% in a second. The maximum variations in individual amino acids ranged from -12 to +9%. This error, however, can be appreciably reduced by increasing the number of chromatograms.

Cystine is largely or entirely destroyed on two-dimensional chromatograms using S. & S. No. 596 or Whatman No. 4 paper under the conditions described above. It is best determined by a combination of the platonic iodide<sup>9</sup> and Fisher<sup>3</sup> procedures. Proline, hydroxyproline and tryptophan are likewise best estimated by special methods, the first two by the isatin method of Fromageot,<sup>10</sup> the last by a combination of the Voisenet-Rhode

\* Experiments which are underway indicate that the concentration of each amino acid on the chromatograms may be estimated within approximately 15% by the maximum color density method, when the color value is read off the appropriate "standard curve" prepared from two dimensional chromatograms which are run *simultaneously* with the unknown solution. As in all colorimetry, it is important to have the color density of the standard as nearly equal as possible to that of the unknown.

<sup>9</sup> Winegard, H. M., Toennies, G., and Block, R. J., *Science*, 1948, **108**, 506.

p-dimethylaminobenzaldehyde reaction (*cf.*<sup>6</sup>) as applied to paper chromatograms and the area<sup>3</sup> or area-density<sup>4</sup> methods.

Methionine,<sup>†</sup> histidine and tyrosine, which are reasonably accurately estimated by specific chromatographic procedures (*cf.* above) have proven to be valuable for the quantitative estimation of amino acids by the ninhydrin method on two-dimensional chromatograms.

**Results.** The values given in Table III show the color ratios and approximate quantities of arginine, histidine, lysine, tyrosine, phenylalanine, methionine, serine, threonine, leucines, valine, glycine, alanine, glutamic acid and aspartic acid obtained on less than 0.3 mg of casein N. The figures given in the adjoining column (Table III) are those calculated from casein by conventional chemical and microbiological methods.<sup>6</sup>

**Summary.** Two methods are described which permit the estimation of colored substances on one-dimensional and two-dimensional paper chromatograms with a minimum expenditure of time and material.

<sup>10</sup> Fromageot, C., private communication, May 1949.

<sup>†</sup> Cystine and methionine have been determined in casein, lactalbumin and human hair hydrolysates by the area method<sup>3</sup> using platonic iodide<sup>9</sup> as the color reagent and water saturated n-butanol-acetic acid as the developing solvent. The following amounts of cystine and methionine, in grams of amino acid per 16.0 g of nitrogen, were found: casein, 0.3; 3.4, lactalbumin 3.0; 2.6, hair 16.0; 0.6.

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## Prophylactic and Therapeutic Effect of Para-Aminobenzoic Acid and Sodium Salicylate on Experimental Allergic Encephalomyelitis.\* (17426)

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### That encephalomyelitis in experimental

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<sup>†</sup> Helen Hay Whitney Foundation, fellow in rheumatic fever.

animals can be produced by the injection of homologous brain tissue was shown by Rivers, Sprunt, and Berry<sup>1</sup> and confirmed by Ferraro

<sup>1</sup> Rivers, T. M., Sprunt, D. H., and Berry, G. P., *J. Exp. Med.*, 1933, **58**, 39.



TABLE III.  
Color Ratios and Amino Acids in Casein.

Amino acid	Standard color ratio	Corrected experimental color ratios (molecular ratio)	Amino acids in casein millimols per 1.60 mg of N	
			Found	Calculated <sup>a</sup>
Arginine	.92	6.4	2.4	2.4
Histidine	1.12	5.1	1.9	2.1
Lysine	.45	14.1	5.4	5.8
Tyrosine	.98	10.0	3.8	3.8
Phenylalanine	.99	9.7	3.6	3.1
Methionine	1.13	6.1	2.3	2.3
Serine	.90	13.2	5.0	5.6*
Threonine	1.02	10.2	3.8	4.0
Leucines	1.08/2	35.8	13.5	12.8
Valine	1.02	15.1	5.7	6.3
Glycine	.89	8.3	3.1	2.8-3.7
Alanine	1.26	9.8	3.7	3.7
Glutamic acid	.72	40.4	15.3	16.1
Aspartic acid	1.33	11.4	4.3	5.6
Proline			++	
Hydroxyproline			—	

\* Uncorrected for hydrolytic losses.

atogram is then developed at right angles to the phenol run with 2,6 lutedine 55 parts, 95% ethanol 20 parts and water 25 parts by volume in an atmosphere of diethylamine and moistened NaCN. At the completion of the run, the chromatograms are dried in the hood by a blast of warm air and the amino acids are revealed by spraying with 0.1 or 0.2% of ninhydrin. The color is brought out by heating the chromatograms. After the color has developed, the maximum color density of each spot is measured with an electronic densitometer (*cf.*<sup>4,5</sup>) using a 5 x 5 mm or a 15.6 mm<sup>2</sup> aperture disc.

**Calculation.** Mixtures of all amino acids in a protein hydrolysate, with the exception of cystine, tryptophan, proline and hydroxyproline, are prepared so as to contain the following quantities of *each* amino acid per ml.: 1.25, 2.50, 5.00 and 10.00 millimols. Twenty-five or more chromatograms are carried out on each of the above standards. The maximum color density of each amino acid on the chromatograms is then determined. The "mean color density", that is the arithmetic mean of the maximum color densities of *all* the spots on each of the 4 standard concentrations, is then calculated. Similarly, the arithmetic means of the color densities given by each individual amino acid on the 4 standard chromatograms is computed. The

latter divided by the former value is the "standard color ratio" of the individual amino acids. "Color ratios" then are calculated in the same manner for all amino acids which are seen on two dimensional chromatograms of acid hydrolysates. These values are called "experimental color ratios." The "standard color ratios" vary less than 10% from run to run over a period of several months and over a range of 1.25 to 10.00 millimols of amino acid per ml. The average "standard color ratios" on S. & S. No. 596 paper are given in Table III.

The "experimental color ratios" of amino acids in the unknown (acid hydrolysate) are determined as above and the values found for each amino acid are then divided by the appropriate "standard color ratio." This gives the "corrected experimental color ratio" of each amino acid on the chromatograms (Table III, 3rd column). The proportion of the "corrected experimental color ratios" to each other gives the molar ratios of each amino acid to the other on the chromatograms. The approximate quantities\* of the amino acids on the chromatograms are then readily determined if the concentration of one, or better 2 or 3, of the amino acids are known from independent chromatographic or other methods.

In those cases where one or more amino

with supplemental vitamin C. Sodium salicylate medications were given by subcutaneous injection of a sterile 5% solution in distilled water. The para-aminobenzoic acid was administered orally with the drinking water in a concentration of 2.5 mg/cc. Sensitization to homologous brain tissue was carried out by subcutaneous injection of a brain-adjuvant (water in oil) emulsion prepared according to our modification of the method of Freund. Ten grams of sterile fresh guinea pig brain were mixed with 100 cc of normal saline by agitation in a Waring Blender. Two parts of this brain emulsion were added to two parts of Bayol F (a light paraffin oil) containing 7.5 mg of heat killed *Mycobacterium butericum* per cc and one part Falba (a Pfaltz and Bauer product containing a mixture of hydroscopic lipids from lanolin.) The final mixture was then agitated with a Waring Blender for 4 periods of 5 minutes each. In this way a stable emulsion was produced, each cc of which contained 2.5 mg of heat killed *Mycobacterium buterium* and 30 mg guinea pig brain. The plan of the first experiment was to begin all medications 8 days prior to the sensitizing injections and to continue the medications for 60 days after the injection of brain and adjuvant. Ten guinea pigs were given para-aminobenzoic acid alone in dosages of .5 g per kilo per day. Twenty were given moderate dosages of salicylate, (.2 g per kilo in one subcutaneous injection each day.) Ten were given larger dosages of salicylate, (.3 g per kilo in divided dosage given 2 times daily), while an additional 20 were treated with a combination of para-aminobenzoic acid (.5 g/K/D) and sodium salicylate (.2 g/K/D). Control groups comprising guinea pigs injected with brain and adjuvants, brain alone, adjuvants alone and adjuvants in addition to salicylate and para-aminobenzoic acid medication were included in the study. Simultaneously, 10 normal untreated guinea pigs fed the same type of rations and given the same routine care as the experimental animals served as additional controls.

In an attempt to determine the therapeutic effect, if any, of sodium salicylate and para-aminobenzoic acid on experimental disseminated encephalomyelitis the second experi-

ment was carried out. Twelve guinea pigs from among 20 weighing 300 to 500 g, which had been inoculated with the brain-adjuvant emulsion previously described, were selected for treatment after the onset of unequivocal central nervous system disease. Four of these diseased animals were treated with sodium salicylate alone in a dosage of .2 g/K/D, and eight were placed on the combined para-aminobenzoic acid and salicylate regimen described for the animals of experiment 1.

In an attempt to establish the time at which para-aminobenzoic acid and salicylate became effective in the prophylaxis of acute disseminated encephalomyelitis, the third experiment was carried out. Forty guinea pigs weighing 425 to 600 g were studied. Ten of these animals injected with the brain adjuvant emulsion and given no further treatment served as controls, while one of the remaining guinea pigs was started on treatment with the para-aminobenzoic acid and salicylate combination previously mentioned on each successive day beginning eight days prior to the injection of all the animals with the brain adjuvant emulsion and continuing for 22 days thereafter.

*Results.* The results of Experiment 1 are summarized in Fig. 1. Approximately 90% of 22 guinea pigs receiving injections of guinea pig brain and adjuvants developed symptoms of central nervous system disease between 13 and 27 days after the injection. Most frequently the disease began with spasticity of the hind legs followed within a few days by flaccid paralysis and encephalitis. Tremors, nystagmus, and convulsions were prominent symptoms in the late stages of the disease. Seventeen of these control animals died from encephalomyelitis between 19 and 42 days after the initial injection of the water in oil emulsion of guinea pig brain. The duration and course of the disease after onset were variable with exacerbations and remissions being noted occasionally. Once unequivocal evidence of central nervous system involvement became apparent, the animals usually died. In several guinea pigs, however, the disease became stationary for long periods. A few animals recovered, some with sequellae,

and Jervis.<sup>2</sup> Studies by Schwentker and Rivers<sup>3</sup> and Kopeloff and Kopeloff<sup>4</sup> demonstrated the association of this disease with the production of specific anti-brain antibodies. Recent studies<sup>5-10</sup> using Freund's adjuvants to potentiate and hasten the development of encephalomyelitis against homologous or heterologous brain tissue have provided a working laboratory model of acute disseminated encephalomyelitis. Almost without exception investigators studying this disease of laboratory animals have supported the allergic theory of its pathogenesis.<sup>11</sup> That the sensitizing agent is concentrated in the white matter of the nervous system, but also present in the gray matter seems clear.<sup>12,13</sup> Alvord's<sup>14</sup> studies have, further, implicated certain purified phospholipids extracted from brain tissue as at least one of the sensitizing hapten groups capable of producing encephalomyelitis, and Bell *et al.*,<sup>15</sup> have recently shown that calcium acetate precipitation of aqueous suspensions of central nervous tissue removes their encephalogenic properties.

In spite of the apparent relation of experimental encephalomyelitis to a group of human diseases (acute disseminated encephalomyeli-

tis, multiple sclerosis) for which effective therapy is lacking, little work has been reported of attempts to find therapeutic or prophylactic agents capable of interrupting the cycle resulting in allergic central nervous system disease in animals. The long recognized clinical usefulness of salicylates in the treatment of the exudative phase of rheumatic fever has led, however, to a series of investigations demonstrating a pronounced effect of large doses of salicylate on the course of antibody production<sup>16-19</sup> and allergic phenomena resulting from antigen-antibody reactions,<sup>20,21</sup> while Coburn and Kapp<sup>22</sup> showed that salicylate could prevent the *in vitro* precipitation of antigen with specific antibody. Suppression of rheumatic symptomatology with paraaminobenzoic acid has been reported from several clinics and the combination of paraaminobenzoic acid with salicylates has been suggested for the treatment of rheumatic fever. Recent studies indicate that both paraaminobenzoic acid and salicylates are protective against certain allergic or hypersensitive states in rabbits<sup>20</sup> and several rather poorly controlled clinical studies indicate that salicylates might be helpful in certain human encephalitis.<sup>23-25</sup>

In an attempt to prevent the development of the acute allergic encephalomyelitis due to immunization against homologous brain tissue, the following 3 experiments were carried out.

In the first experiment 112 guinea pigs weighing 250 to 325 g each were used. All animals were fed balanced guinea pig rations

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<sup>3</sup> Schwentker, F. F., and Rivers, T. M., *J. Exp. Med.*, 1934, **60**, 559.

<sup>4</sup> Kopeloff, L. M., and Kopeloff, N., *J. Immunol.*, 1944, **48**, 297.

<sup>5</sup> Morgan, I. M., *J. Bact.*, 1946, **51**, 53.

<sup>6</sup> Kabat, E. A., Wolf, A., and Bezer, A. E., *Science*, 1946, **104**, 362.

<sup>7</sup> Freund, J., Stern, E. R., and Pisani, T. M., *J. Immunol.*, 1947, **57**, 179.

<sup>8</sup> Morrison, L. R., *Arch. Neurol. and Psychiat.*, 1947, **58**, 391.

<sup>9</sup> Jervis, G., and Koprowski, A., *J. Neuropath. and Exp. Neurol.*, 1948, **7**, 309.

<sup>10</sup> Ferraro, A., and Cazzullo, C. L., *J. Neuropath. and Exp. Neurol.*, 1948, **7**, 235.

<sup>11</sup> Stevenson, L. D., and Alvord, E. C., *Am. J. Med.*, 1947, **3**, 614.

<sup>12</sup> Kabat, E. A., Wolf, A., and Bezer, A. E., *J. Exp. Med.*, 1947, **85**, 117.

<sup>13</sup> Morgan, I. M., *J. Exp. Med.*, 1947, **85**, 131.

<sup>14</sup> Alvord, E. C., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 459.

<sup>15</sup> Bell, N. F., Wright, J. T., and Habel K., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 457.

<sup>16</sup> Swift, H. F., *J. Exp. Med.*, 1922, **36**, 735.

<sup>17</sup> Derriek, C. L., Hitchcock, C. H., and Swift, H. F., *J. Clin. Invest.*, 1928, **5**, 427.

<sup>18</sup> Jager, B. V., *Proc. Am. Fed. Clin. Res.*, 1947, **3**, 93.

<sup>19</sup> Homburger, F., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 101.

<sup>20</sup> Sullivan, C. J., Parker, T. W., and Hibbert, R. W., *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 508.

<sup>21</sup> Campbell, B., *Science*, 1948, **108**, 478.

<sup>22</sup> Coburn, A. F., and Kapp, E. M., *J. Exp. Med.*, 1943, **77**, 173.

<sup>23</sup> Maciel, Z., *Brazil med.*, 1941, **55**, 804.

<sup>24</sup> Behague, P., *Rev. neurol.*, 1942, **74**, 159.

<sup>25</sup> Ferraz, A. J., *Rev. neurol. e psiquiatr. de São Paulo*, 1944, **10**, 1.

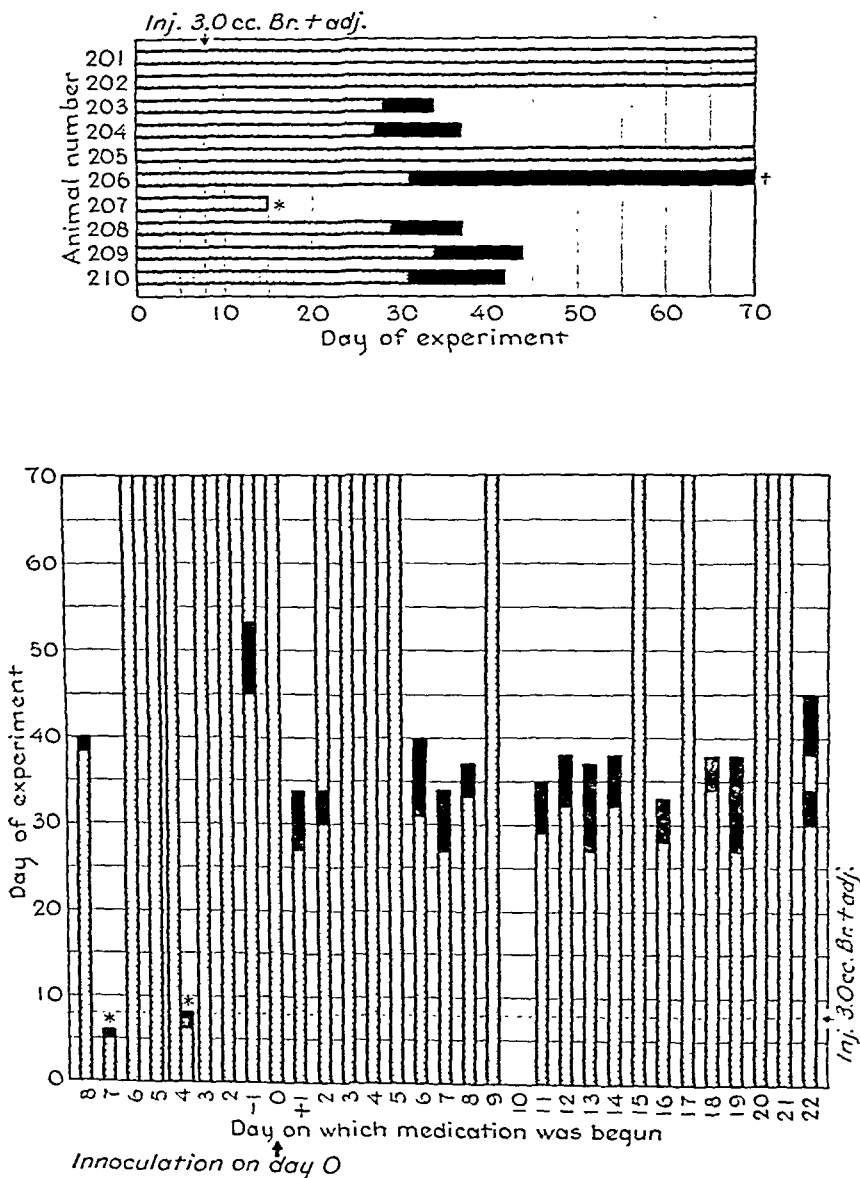


FIG. 2.

Chart showing individual clinical histories of 10 control animals and 30 which commenced medication at various days, before and after the sensitizing injection. Stippled columns indicate disease-free period, solid columns represent period of illness. Termination of the columns indicates death of animal. Asterisks indicate death of animals from causes other than the experimental disease. Animal 206, long sick, was killed at the termination of the experiment.

salicylate alone died between 4 and 18 days after the initial symptoms and beginning of treatment. This result was not significantly different from that occurring among the control guinea pigs.

The results obtained in Experiment 3 are indicated in Fig. 2. Here again evidence of a protecting effect of combined para-amino-benzoic acid and salicylate prophylaxis is apparent. The control group (Fig. 2) which

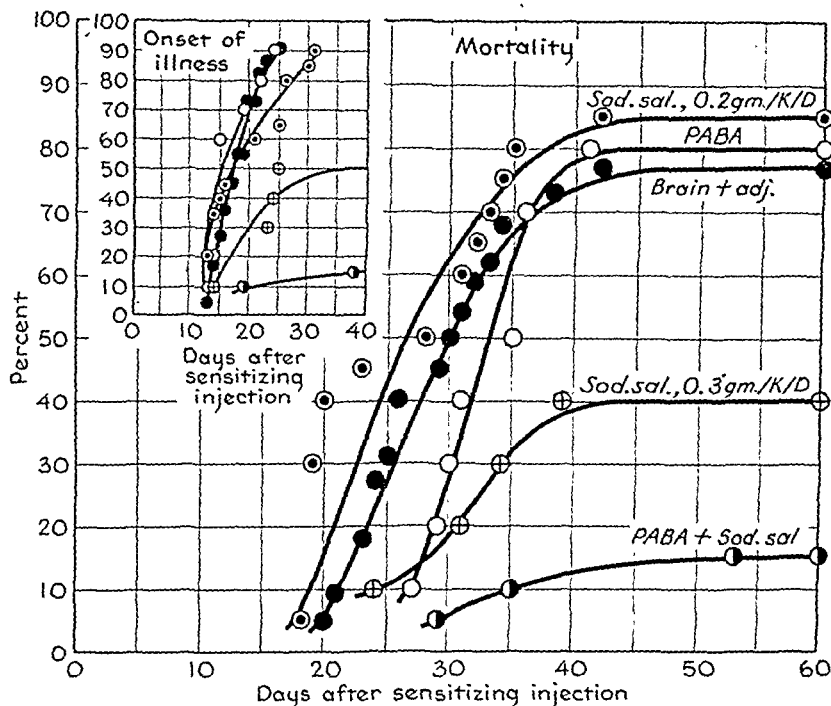


FIG. 1.

Table of mortality curves of 112 guinea pigs of experiments I and II. Insert shows onset of illness in same series. See text.

others with none. Cultures of the central nervous tissue removed with careful aseptic precautions from moribund, paralysed, encephalitic animals showed no growth on blood agar plates. Neither were attempts to demonstrate a virus in the involved nervous tissue by intracranial and intraperitoneal mouse passage, intracranial and intraperitoneal guinea pig passage, nor corneal and intraocular rabbit passage successful.

While premedication and medication with moderate doses of either sodium salicylate or para-aminobenzoic acid alone failed to alter the incidence or the course of the central nervous system disease, larger total dosages of sodium salicylate seemed to delay the time of onset and reduce both the incidence and mortality rate. Even more striking were the results obtained when a combination of para-aminobenzoic acid and sodium salicylate was used for the prophylaxis and treatment of this experimental disease. Fifteen per cent of the guinea pigs treated in this way developed symptoms and signs of encephalomyelitis after

injection with brain and adjuvant and only 10% died. Injection of either adjuvants or homologous brain alone was well tolerated by the controls. During the period of the experimental study, 10 normal guinea pigs remained in good health showing substantial growth on the same dietary regimen provided for the experimental animals.

The therapeutic effectiveness of sodium salicylate and para-aminobenzoic acid was tested on a group of 12 guinea pigs selected on the first day of central nervous system involvement following 14-30 days after the injection of brain and adjuvants. No alteration in the course of the disease nor apparent amelioration of symptoms was produced in any of the animals treated with sodium salicylate or combined sodium salicylate and para-aminobenzoic acid. One guinea pig in the group treated with para-aminobenzoic acid and salicylate showed several remissions followed by exacerbations and ultimately survived. The remaining seven guinea pigs of this group and all 4 treated with sodium

Para-aminobenzoic acid may by itself have anti-anaphylactic properties<sup>20</sup> and the effect noted here might be an expression of a synergistic antianaphylactic action. It is entirely possible, however, that the decreased elimination of sodium salicylate in the urine in the presence of para-aminobenzoic acid<sup>23</sup> results in higher, more effective, blood levels of salicylate than when this drug is given alone. If so, other acid salts, like ammonium chloride, might also result in a comparable enhancement of the salicylate effect. While toxic reactions to salicylates and para-aminobenzoic acid may preclude the safe attainment of effective levels in the treatment of human disease, further studies are indicated to elucidate the underlying mechanisms of this protecting effect. There is reason from this and other studies to believe that a less toxic drug with antianaphylactic properties in common with salicylates might be an important therapeutic and prophylactic agent in human disease.

The now extensive studies of auto-immunization phenomena in diseases of experimental animals demand further attempts to establish the operation of such mechanisms in the pathogenesis of human disease in spite of the technical difficulties involved. It seems obvious that this phenomenon could be at work

in post infectious states, the pathogenic mechanisms of which remain obscure. Further studies on rheumatic fever, post infectious leukoencephalitis, multiple sclerosis, sympathetic ophthalmia, and glomerulonephritis might, indeed, reveal that autosensitization is a common denominator for many otherwise unrelated diseases.

*Summary and conclusions.* 1. Progressive acute disseminated encephalomyelitis was produced in 90% of a group of guinea pigs by the subcutaneous injection of water in oil emulsions of homologous brain tissue plus adjuvants.

2. While prophylaxis and treatment with moderate dosages of either para-aminobenzoic acid or sodium salicylate alone provided no protection of guinea pigs against experimental allergic encephalomyelitis, the use of larger dosages of salicylate as well as combined salicylate and para-aminobenzoic acid therapy were effective in preventing this disease.

3. Effective prophylaxis was demonstrated only when the medication was started before or shortly after the sensitizing injection of brain tissue was given.

4. No therapeutic effect of sodium salicylate alone or combination of para-aminobenzoic acid with sodium salicylate could be shown.

<sup>23</sup> Salassa, R. M., and Bollman, J. L., *Am. J. Physiol.*, 1948, 155, 466.

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## The Initial Uptake of C<sup>14</sup>-Labelled Methadone by Rat Tissues.\* (17427)

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Recently it has been shown in this laboratory that within one hour after a subcutaneous injection of 10 mg/kg of C<sup>14</sup> labelled methadone (methadone\*) into rats, the concentration of the drug in the brain is relatively

low.<sup>1</sup> A fairly rapid mobilization of the drug from the site of injection is indicated by the work of Eisenbrandt, *et al.*<sup>2</sup> who found that within 10 minutes after such an injection,

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† On sabbatical leave from the University of Kansas City, Kansas City, Mo.

<sup>1</sup> Elliott, H. W., Chang, F. N.-H., Abdou, I. A., and Anderson, H. H., *J. Pharm. and Exp. Therap.*, 1949, 95, 494.

<sup>2</sup> Eisenbrandt, L. L., Abdou, I. A., and Adler, T. K., *Fed. Proc.*, 1949, 8, 288.

received brain plus adjuvant but no medication, showed 5 out of 9 dying (55%) with an incidence of 6 out of 9 acquiring the disease (67%). In the treated series, little morbidity was seen in the group in which medication was commenced before the fifth day following injection. Past that point, however, the incidence of symptoms and death appear not significantly different from the control. These results suggest that protection against experimental allergic encephalomyelitis is effective when sodium salicylate and para-aminobenzoic acid medication are begun early. It would seem from these data that if medication is delayed for several days following the injection of the brain and adjuvant no protecting effect can be demonstrated. In this series significant protection was apparent among the guinea pigs whose treatment was initiated prior to the fifth day following injection with brain and adjuvant, while those whose treatment was begun later were not protected.

Although the pathologic studies of the series of animals will be the subject of another report, a brief mention of the results obtained are of interest here. The central nervous system changes include scattered foci of inflammatory reaction with subependymal and perivascular concentrations being characteristic. The marked plasmacellular component of the inflammatory reaction previously noted by others<sup>8-10</sup> was also apparent in our material and is in keeping with an hypothesis previously expressed<sup>26-28</sup> that local plasma cell accumulation in the central nervous tissue, as well as elsewhere, indicates the operation of antigen-antibody reactions.

**Discussion.** Many problems are posed by these results. Although in these experiments serum antibodies were not studied, we assume that the disease with which we are dealing is an expression of the specific antibrain hypersensitivity. A number of investigators have

demonstrated the association of the nervous disease produced in this way with the development of antibrain antibodies.<sup>3,4,32</sup> Because of our previous experience<sup>29,30</sup> with the activation of latent virus in the central nervous system by antigen-antibody reaction and because the symptoms of nervous system involvement in this disease simulate in certain respects known virus disease of guinea pigs, mechanisms involving virus invasion were suspected of operating in this situation. It seems reasonably certain, however, that our futile search for virus in the tissues of these animals as well as negative searches for virus in the central nervous tissues of similar animals by others render this possibility very unlikely. The constant two to four week latent period following injection of antigen is compatible with the allergic hypothesis of the pathogenesis of this encephalomyelitis.

The protection afforded by the combination of salicylates and para-aminobenzoic acid can probably be explained through operation of known actions of these drugs. An *in vivo* as well as *in vitro* effect of salicylates in blocking antigen-antibody reactions seems well established. In addition, the weight of the evidence would indicate that large doses of salicylates act in some way to reduce antibody formation. That moderate dosages of salicylates do not affect antibody response to the streptococcus and its products, was pointed out by Rantz, Boisvert, and Spink<sup>31</sup> while the effect of larger dosages of salicylate on the antibody response to a variety of antigens has been demonstrated. This relationship might help to explain the lack of protection against acute allergic encephalitis noted when sodium salicylate in a dosage of .2 g/K/D was used while protection was obtained with larger dosage of salicylate as well as when the smaller salicylate dosage was combined with para-aminobenzoic acid.

<sup>29</sup> Good, R. A., and Campbell, B., *Proc. Soc. Exp. Biol. and Med.*, 1945, **59**, 305.

<sup>30</sup> Good, R. A., and Campbell, B., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 82.

<sup>31</sup> Rantz, L. A., Boisvert, P. J., and Spink, W. W., *Science*, 1946, **103**, 352.

<sup>32</sup> Kuprowski, H., and Jervis, G. A., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 472.

<sup>26</sup> Campbell, B., *J. Neuropath. and Exp. Neurol.*, 1949, **8**, 347.

<sup>27</sup> Good, R. A., *J. Neuropath. and Exp. Neurol.*, in press.

<sup>28</sup> Campbell, B., and Good, R. A., *Ann. Allergy.*, 1949, **7**, 471.

TABLE I.  
Tissue Concentration of Radioactivity in Terms of Micrograms of Methadone\* per Gram.

Rat No.	Time after inj., min.	Cerebrum		Plasma		Adrenals		Liver	
		per g water	per g dry tissue	per g water	per g dry tissue	per g water	per g dry tissue	per g water	per g dry tissue
16	10	0	0	0.4	4.9	0	0	2.4	5.4
21	10	0	0	0.05	5.5	11.2	16.9	4.1	9.5
17	20	2.3	8.3	0.9	10.5	16.0	42.8	18.8	49.2
22	20	6.0	20.0	2.6	27.9	39.7	68.4	10.9	25.7
18	30	0	0	0.9	9.8	22.9	26.4	20.1	47.5
23	30	3.3	11.9	1.2	14.5	27.4	51.5	13.4	33.6
19	40	3.6	13.0	2.0	24.9	48.5	93.0	25.5	60.7
24	40	2.5	8.6	2.5	30.0	45.0	96.5	30.8	79.4

induced by an initial high concentration of the drug in the brain.

In contrast to the cerebrum, both the liver and adrenals showed a marked increase in concentration with time; the rate of increase in the adrenals was more pronounced. These differences, obtained even when calculated on the basis of tissue water, suggest a definite mechanism for accumulation in these tissues.

*Summary.* Based on the determination of radioactivity for periods of 10, 20, 30, and 40 minutes following a subcutaneous injection of 10 mg./kg of C-<sup>14</sup> labelled methadone HBr into rats, it was found that:

(a) Methadone\* makes its appearance in

the blood within 10 minutes, and is confined to the plasma.

(b) There was no activity in the cerebrum before 10 minutes, and even thereafter the concentration is very low. The rate of accumulation roughly parallels that of the plasma indicating no special affinity of the brain for the drug.

(c) The adrenals and liver show a marked ability to accumulate methadone\* at a rate in excess of that shown by either the cerebrum or plasma.

(d) There was no activity in the sciatic nerve within the period studied.

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## A Nervous Syndrome Produced with Phenylalanine and Methionine.\* (17428)

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During studies on the metabolic interrelationships between threonine, phenylalanine, tryptophan and niacin, it was noted that animals fed a niacin-tryptophan deficient ration supplemented with 0.208% DL-phenyl-

alanine and 0.2% DL-methionine for a period of 12 weeks developed an interesting nervous syndrome. The symptoms include an arched back with extended legs in a rigid condition. The head oscillates vertically almost continuously, and the rate of movement increases until the animal apparently becomes fatigued. After a short rest period, this movement re-occurs. The animal may show a slow rotary motion of the body and periodically it may move backwards at a rapid rate. Death re-

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radioactivity appeared in the bile. In view of this rapid mobilization it was desirable either to establish or to rule out the possibility that an initial high concentration of methadone in the brain is responsible for the rather profound effects on the central nervous system.

The study was extended to include determination of the rate of accumulation of methadone\* by the adrenals and the liver, both of which were found by Elliott, *et al.*,<sup>1</sup> to contain high concentrations after one hour, and to correlate these findings with the rate of accumulation in the blood. In addition, portions of the sciatic nerve were analyzed to check the possibility that analgesia might be produced by concentration of methadone\* in the peripheral nervous system.

**Methods.** The C-<sup>14</sup> labelled methadone HBr used in this study has been described previously.<sup>1,3</sup> Eight young adult rats (4 male and 4 female) of the Slonaker-Wistar strain weighing between 175-250 g and maintained on a diet of Purina Dog Chow and tap water were used in this study. Each was given 10 mg/kg of methadone\* by subcutaneous injection into the left hind leg. After 10, 20, 30, or 40 minutes one male and one female were sacrificed. Ether was administered immediately before sacrifice, and a blood sample was obtained from the abdominal aorta.

Cerebrum, adrenals, liver and portions of the sciatic nerve from the uninjected (right) leg were dried to constant weight at 60°C. Tissue water content was calculated from the wet-dry weight difference.

Preparation of tissues for combustion has been described previously<sup>1</sup>. The method of combustion was that of Van Slyke and Folch<sup>4</sup> as modified by Skipper, *et al.*<sup>5</sup> The combustion

apparatus used has been described by Entenman, *et al.*<sup>6</sup> Preparation of radioactive samples of BaCO<sub>3</sub> for counting was similar to that used previously in this laboratory.<sup>1</sup>

Although there is evidence that methadone can be metabolized by the body,<sup>7,8</sup> the possible metabolic products have not been identified. However, the labelled carbon atom incorporated in the methadone\* used in these studies was not oxidized to CO<sub>2</sub>.<sup>1</sup> Therefore, for the sake of simplicity the data are reported as micrograms of methadone\*.

**Results.** A comparison of the concentration of radioactivity in whole blood with that in the plasma indicated a dilution of the former by the red blood cells. To establish this point definitely, the red blood cells were centrifuged, washed twice with saline and analyzed. No radioactivity could be found, thus indicating that within the period studied (between 10 and 40 minutes) the methadone\* in the blood was confined to the plasma.

Furthermore, it seemed desirable to establish whether or not accumulation of the drug in the tissues was merely a reflection of accumulation in the plasma water. For this reason calculations were made on the basis of concentration per gram of tissue water in addition to the usual method of reporting such data.

The concentration of methadone\* in the tissues studied is summarized in Table I. Values for the sciatic nerve are omitted from the table since at no time did the nerve contain significant amounts of radioactivity.

**Discussion.** The rapid appearance of radioactivity in the plasma indicates the importance of the plasma as a transport mechanism in mobilizing a subcutaneous injection of methadone\*. It is of interest to note that the concentration in the cerebrum was of the same order of magnitude as that of the plasma, and also that the drug did not accumulate in the sciatic nerve. The low amounts in the cerebrum dispute the thesis that analgesia is

<sup>3</sup> Tolbert, B. M., Christenson, F., Chang, F. N.-H., and Sah, P. P. T., *J. Am. Chem. Soc.*, in press.

<sup>4</sup> Van Slyke, D. D., and Folch, J., *J. Biol. Chem.*, 1940, **136**, 509.

<sup>5</sup> Skipper, H. E., Bryan, C. E., White, L., Jr., and Hutchinson, O. S., *J. Biol. Chem.*, 1948, **173**, 371.

<sup>6</sup> Entenman, C., Lerner, S. K., Chaikoff, I. L., and Dauben, W. G., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 364.

<sup>7</sup> Richards, J. C., and Boxer, G. E., paper presented to the Division of Medicinal Chemistry of the Am. Chem. Soc. meeting of Sept. 18-23, 1949.

<sup>8</sup> Way, E. L., Signorotti, B. T., and March, C. H., to be published.

The apparent inability of the animals to control their movements indicated that the cells in certain areas of the brain may have been affected. The levels of methionine and phenylalanine in the rations may have altered the ratio of amino acids in the brain cells<sup>2</sup> to such an extent that permanent changes in the enzyme or metabolic systems of the cells took place. Furthermore, the observation that the symptoms were not reversible by feeding or injecting niacin would substantiate this conclusion, since this compound prevented the onset of the nervous symptoms, when it was present in the ration. A gross examination of the brains of several animals did not reveal any abnormalities.

It is believed that this is the first time a syndrome of this nature has been observed in experiments with rations containing an imbalance of amino acids. There are known human diseases such as cystinuria,<sup>3,4</sup> alkaptonuria,<sup>5</sup> tyrosinosis,<sup>6</sup> and phenylpyruvic oligophrenia,<sup>7,8</sup> which have been related to inborn errors of amino acid metabolism. In

these diseases normal metabolic processes involving phenylalanine and tyrosine are either partially or completely inhibited, and the disorders are generally recognized by the persistent excretion of abnormal urinary constituents.<sup>9,10</sup>

When our results are considered in the light of faulty amino acid metabolism, it is feasible that the effects observed were due partially to a limiting ability of the animal to metabolize phenylalanine. It is also possible that the causative agent in this syndrome was the D isomer of this amino acid, since it has been observed that the growth of rats was reduced drastically when this isomer was supplemented at low levels in a 9% casein ration.<sup>1</sup>

**Summary.** Animals fed a niacin-tryptophan deficient ration supplemented with 0.208% DL-phenylalanine and 0.2% DL-methionine for a period of 12 weeks developed a nervous syndrome. Generous quantities of tryptophan or niacin in the ration protected the animals from this syndrome, but neither compound cured the symptoms once they had progressed beyond a given stage.

We are indebted to Merek and Co., Inc., Rahway, N. J., for the supply of crystalline B vitamins, and to the Abbott Laboratories, North Chicago, Ill., for the generous supply of haliver oil.

<sup>9</sup> Sealock, R. R., and Silberstein, H. E., *J. Biol. Chem.*, 1940, **135**, 251.

<sup>10</sup> Basinski, D. H., and Sealock, R. H., *Fed. Proc.*, 1946, **5**, 121.

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<sup>2</sup> Christensen, H. N., Streicher, J. A., Elbinger, R. L., *J. Biol. Chem.*, 1948 **172**, 515.

<sup>3</sup> Burke, B. S., Harding, V. V., and Stuart, H. C., *J. Pediat.*, 1943, **23**, 508.

<sup>4</sup> Lewis, H. B., Brown, B. H., and White, F. R., *J. Biol. Chem.*, 1935, **109**, 69.

<sup>5</sup> Hall, W. K., Rawls, K., and Sydenstricker, V. P., *Fed. Proc.*, 1946, **5**, 136.

<sup>6</sup> Medes, G., *Biochem. J.*, 1932, **26**, 917.

<sup>7</sup> Dann, M., Marples, E., and Levine, S. Z., *J. Clin. Invest.*, 1943, **22**, 87.

<sup>8</sup> Jarvis, G. A., Block, R. J., Bolling, D., Kanze, E., *J. Biol. Chem.*, 1940, **134**, 105.

## A Modified Diphenylamine Procedure for Fructose or Inulin Determination. (17429)

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The use of inulin clearance as a measure of glomerular filtration rate has prompted

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the appearance of a number of methods for the determination of inulin or fructose, the majority of which are based either on the Seliwanoff reaction<sup>1</sup> or the reaction with

sults 36 to 50 hours after the onset of the symptoms. Blood pressure measurements on one animal with very severe symptoms gave normal values. Autopsy of all animals failed to reveal any gross abnormalities in the structure of any of the organs; however, the stomachs of some were very slightly bloated.

The animals fed niacin or tryptophan in the ratio in addition to the added DL-phenylalanine and DL-methionine were normal. The rations supplemented with 0.2% L-cystine plus 0.208% DL-phenylalanine instead of 0.2% DL-methionine plus 0.208% phenylalanine did not produce this syndrome. Even though DL-threonine is generally much more active as a growth inhibitor in the 9% casein ration, it did not produce these symptoms, when either L-cystine or DL-methionine was added to the ration. Apparently there is a definite relationship between methionine and phenylalanine which is responsible for the nervous syndrome.

An experiment was designed (Table I) to determine what levels of methionine and phenylalanine would be required to shorten the period for onset of the syndrome, and what compounds could be used to cure it. The composition of the basal ration was the same as that used in previous studies.<sup>1</sup> The combinations of 0.208% DL-phenylalanine plus 0.2% DL-methionine, 0.208% DL-phenylalanine plus 0.4% DL-methionine and 0.416% DL-phenylalanine plus 0.4% DL-methionine all appeared to produce the nervous syndrome in the same period of time. The administration of niacin or tryptophan by stomach tube, the injection of niacin, or the feeding of tryptophan solution orally by dropper, after the animals exhibited severe nervous symptoms, did not produce a cure. On the other hand if the level of casein in the ration was doubled at the onset of the early symptoms of the syndrome, the animals quickly recovered. It is important that niacin or tryptophan protected the animals when given from the beginning, but they were inactive when given after the syndrome had reached a definite stage.

TABLE I. Response of Individual Rats to Various Supplements Administered After Onset of Nervous Syndrome.

Supplement	Time required for symptoms to appear, wk	Survival time after appearance of symptoms, hr	Treatment
0.208% DL-Phenylalanine + 0.2% DL-methionine	12	56	No treatment.
"	12.5	50	100 mg DL-tryptophan in 25% alcohol sol. fed (ml containing 20 mg) by stomach tube 48 hr. 1 ml fed every 8 hr.
"	7	24	No treatment.
"	9	60	12.5 mg niacin in 25% alcohol sol. fed (ml containing 12.5 mg) by stomach tube. After 24 hr period, 8 mg niacin (8 mg/ml) in aqueous sol. inj. 18% casein ration fed; complete recovery followed.
"	11	—	No treatment.
"	3	24	No treatment.
"	8	36	No treatment. Blood pressure normal (125 mm).
"	11	—	Brain removed for pathological studies.
"	9	36	18% casein ration fed; complete recovery followed.
0.416% DL-Phenylalanine + 0.4% DL-methionine	11	—	25 mg DL-tryptophan in 25% alcohol fed by dropper 48 hr. At autopsy all tissues appeared normal.
"	—	—	18% casein ration fed; complete recovery followed.
"	—	—	The rat removed at end of 13 wk.

<sup>1</sup> Hanks, L. V., Henderson, L. M., Brickson, W. L., and Elvehjem, C. A., *J. Biol. Chem.*, 1948, 174, 873.

TABLE I.  
 Optical Densities  $\times 10^3$  Produced by Fructose, Inulin, and Glucose Solution.

	Fructose, $\mu\text{g}/2 \text{ ml}$					Glucose, mg/2 ml 1	Fructose glucose
	10	20	30	40	60		
1		$145 \pm .5(6)$	$222 \pm .6(6)$	$289 \pm 1.0(6)$	$433 \pm 1.0(6)$		
2		$142 \pm .5(5)$	$220 \pm 1.0(6)$	$285 \pm .9(6)$	$436 \pm .8(6)$	$117 \pm .7(4)$	62
3	$89 \pm .5(5)$	$147 \pm .5(5)$	$221 \pm .7(5)$	$291 \pm .8(5)$	$447 \pm .5(5)$	$114 \pm .6(4)$	65
4	$82 \pm .3(6)$	$154 \pm .5(5)$	$236 \pm .2(5)$	$309 \pm .9(4)$	$460 \pm .4(4)$	$124 \pm .5(3)$	63
5	$77 \pm .5(3)$	$148 \pm .7(3)$	$220 \pm .7(3)$	$294 \pm 1.0(3)$	$448 \pm .5(3)$	$118 \pm .0(2)$	63
6	$74 \pm .5(3)$	$152 \pm .4(3)$	$228 \pm 2.0(3)$	$295 \pm 1.0(3)$	$445 \pm 1.0(3)$	$114 \pm 1.0(3)$	66
7	$72 \pm .0(3)$	$148 \pm .7(3)$	$230 \pm 2.0(3)$	$305 \pm 1.0(3)$	$457 \pm 2.0(3)$	$120 \pm 1.0(3)$	63
Inulin $\mu\text{g}$ per 2 ml.							
8			$229 \pm .7(10)$				
9		$142 \pm .7(6)$	$204 \pm .4(6)$	$284 \pm .7(4)$	$426 \pm 1.0(6)$		

The data of a horizontal line were obtained with a single heating. The figure following the  $\pm$  is S.E. for the samples of a given concentration in the same heating; the figures in parentheses give  $n$ , the number of samples. Ten 30  $\mu\text{g}$  fructose samples run simultaneously with the 10 inulin samples of line 8 gave an average optical density  $\times 10^3$  of  $234 \pm 0.8$ , indicating complete hydrolysis of the inulin. The inulin densities in line 9 are somewhat lower than those usually obtained because some diphenylamine had precipitated after the reagent had stood 2 days in the refrigerator; simultaneous fructose determinations (not shown in table) were also correspondingly low.

60 ml of conc. HCl. This is the same reagent used by Harrison. Prepare fresh each day; if kept in a refrigerator some of the diphenylamine will precipitate and it is difficult to redissolve it. Use of this reagent after partial precipitation of diphenylamine yields a calibration curve which is lower than one obtained with fresh reagent.

**Procedure.** Pipette 2 ml of a 1:10 plasma filtrate<sup>7</sup> or appropriate urine dilution into a test tube 15 x 125 mm. Add 4 ml of diphenylamine reagent and *mix well* with swirling. Since the reagent has a high viscosity, care must be taken that pipette drainage is adequate. For plasma determinations use as a blank 2 ml of a 1:10 filtrate of plasma obtained just prior to the injection of inulin; 2 ml of water is used for urine blanks. Place in a rack and heat in a water bath at 75°C for 60 minutes. The bath used had a thermoregulator sensitive to  $\pm 0.7^\circ\text{C}$  at 75°C; a mechanical stirrer insured uniform heating of all samples. At the end of the heating period place rack in an ice bath for 1 to 2 minutes. If the tubes are kept too long at the lower temperature there

may be some precipitation of diphenylamine. Read the samples in a photoelectric colorimeter with a 640 filter or a spectrophotometer at 640  $m\mu$ .

When 2 ml samples containing 10 to 60  $\mu\text{g}$  of fructose or inulin in water solution are used, optical density plotted against concentration yields a straight line. This corresponds to a range from 5 to 30 mg per 100 ml of plasma since 2 ml of a 1:10 filtrate are used. Separate calibration curves may not superimpose. Since the curves are straight lines, the inclusion of a standard with each set of determinations makes it possible to correct the values read from the calibration curve.

Table I shows the optical densities obtained with various concentrations of fructose, inulin and glucose. There is good agreement between samples of the same concentration in any one heating batch (represented on a horizontal line). Within the range of concentrations shown in Table I a given amount of fructose yields 64 times as much color as the same amount of glucose; this same ratio holds when fructose color development is compared with that of glucose in concentrations of 0.125 and 0.250 mg/ml (not shown in table). In our experience the plasma glucose level of fasted subjects does not depart more than 10 mg % from the blank value during

<sup>†</sup> Since this paper went to press we have been using 2.5 g of diphenylamine and have been keeping the tubes covered while they are in the water bath. This has prevented crystallization of diphenylamine.

<sup>7</sup> Somogyi, M., *J. Biol. Chem.*, 1930, 80, 655.

diphenylamine described by Ihl.<sup>2</sup> The hexoses when heated in the presence of strong acids form hydroxymethylfurfural and this in turn reacts to form a colored condensation product with either resorcinol or diphenylamine. Although the ketohexoses react much more readily than the aldohexoses, glucose in the concentrations normally found in plasma will yield a significant amount of color. If the plasma glucose remains constant this error is eliminated by the use of a control blank. Since this is not generally the case, the usual procedure has been either to remove the glucose by fermentation or to determine it in each sample and make a correction.

The amount of color produced by fructose or inulin as compared to that produced by glucose varies somewhat with different methods. Factors which influence this ratio are, (1.) acidity of the reacting solution, (2.) concentration of resorcinol or diphenylamine, (3.) duration of heating and (4.) temperature at which the reaction is carried out. While a decrease in any of these factors will decrease the speed of the reaction, the glucose color production is diminished to a greater extent than the fructose. Corcoran and Page,<sup>3</sup> using a diphenylamine procedure at 100°C, found a fructose to glucose color ratio of 40 and say, "Error from this source is abolished by the compensating blank when blood glucose is constant, and the error amounts to only 0.5 mg of levulose per 100 cc with glucose concentrations varying 20 mg per 100 cc." Using their method we have found the ratio to be between 20 and 25; Corcoran has recently informed us that they now find this lower ratio. Findley and White<sup>4</sup> found a ratio of 25; since then it has been found to be more nearly 20. Harrison,<sup>5</sup> who has improved the technic by substituting glacial acetic acid for ethyl alcohol as the solvent for diphenylamine, does not state his ratio, but says, "It is

necessary to remove glucose from the blood plasma." Using his method we have found the ratio to be 8. Roe, Epstein and Goldstein,<sup>6</sup> using a resorcinol method, have recently reported a fructose to glucose color ratio of about 2000 at various concentrations of glucose; such a ratio would render any errors due to glucose fluctuations insignificant. We have not been able to confirm their finding. Using their method and working with aqueous glucose solutions containing 0.0625 mg/ml to 0.5 mg/ml and with fructose solutions containing 0.005 mg/ml to 0.03 mg/ml we have found the color ratio to be approximately 50. Color productions by glucose and by fructose are additive when the two sugars are determined together. We have found also that the color produced by this reaction is quite sensitive to light, either artificial or daylight, and that there can be a difference of as much as 15% between duplicates even when the samples are read within the 30 minutes recommended in their paper, unless special precautions are taken to maintain light exposure at a minimum and uniform for all samples.

In an effort to find a set of conditions which would yield a high fructose to glucose color ratio we have tried many combinations of diphenylamine and HCl with different heating times and temperatures. The following procedure has produced the highest fructose to glucose ratio and yielded the best agreement between duplicates. Although we have not been able to verify Roe, Epstein and Goldstein's exceedingly high color ratio, it still appears to be a satisfactory method since even a ratio of 50 is higher than with previously reported methods; in our hands it has yielded accurate results when colored samples are protected from light. It is still felt, however, that the method presented here has some advantages in that the color ratio is somewhat higher and the color produced is stable. It further avoids the unpleasantness involved in handling their highly concentrated HCl reagent.

*Method. Reagent.* Dissolve 3 g of diphenylamine<sup>1</sup> in 100 ml of glacial acetic acid and add

<sup>1</sup> Selivanoff, T., *Ber. chem. Ges.*, 1897, 1, 181.

<sup>2</sup> Ihl, A., *Chem. Ztg.*, 1885, 9, 451.

<sup>3</sup> Corcoran, A. C., and Page, I. H., *J. Biol. Chem.*, 1939, 127, 601.

<sup>4</sup> Findley, T., and White, H. L., *Proc. Soc. Exp. Biol. and Med.*, 1940, 45, 623.

<sup>5</sup> Harrison, H. E., *Proc. Soc. Exp. Biol. and Med.*, 1942, 49, 111.

<sup>6</sup> Roe, J. H., Epstein, J. H., and Goldstein, N. P., *J. Biol. Chem.*, 1949, 178, 839.

TABLE I  
 Optical Densities  $\times 103$  Produced by Fructose, Inulin, and Glucose Solution.

	Fructose, $\mu\text{g}/2\text{ ml}$					Glucose, mg/2 ml 1	Fructose glucose
	10	20	30	40	60		
1		145 $\pm$ .5(6)	222 $\pm$ .6(6)	289 $\pm$ 1.0(6)	433 $\pm$ 1.0(6)		
2		142 $\pm$ .5(5)	220 $\pm$ 1.0(6)	285 $\pm$ .9(6)	436 $\pm$ .8(6)	117 $\pm$ .7(4)	62
3	89 $\pm$ .5(5)	147 $\pm$ .5(5)	221 $\pm$ .7(5)	291 $\pm$ .8(5)	447 $\pm$ .5(5)	114 $\pm$ .6(4)	65
4	82 $\pm$ .3(6)	154 $\pm$ .5(5)	236 $\pm$ .2(5)	309 $\pm$ .9(4)	460 $\pm$ .4(4)	124 $\pm$ .5(3)	63
5	77 $\pm$ .5(3)	148 $\pm$ .7(3)	220 $\pm$ .7(3)	294 $\pm$ 1.0(3)	448 $\pm$ .5(3)	118 $\pm$ .0(2)	63
6	74 $\pm$ .5(3)	152 $\pm$ .4(3)	228 $\pm$ 2.0(3)	295 $\pm$ 1.0(3)	445 $\pm$ 1.0(3)	114 $\pm$ 1.0(3)	66
7	72 $\pm$ .0(3)	148 $\pm$ .7(3)	230 $\pm$ 2.0(3)	305 $\pm$ 1.0(3)	457 $\pm$ 2.0(3)	120 $\pm$ 1.0(3)	63
Inulin $\mu\text{g}$ per 2 ml.							
8			229 $\pm$ .7(10)				
9		142 $\pm$ .7(6)	204 $\pm$ .4(6)	284 $\pm$ .7(4)	426 $\pm$ 1.0(6)		

The data of a horizontal line were obtained with a single heating. The figure following the  $\pm$  is S.E. for the samples of a given concentration in the same heating; the figures in parentheses give  $n$ , the number of samples. Ten 30  $\mu\text{g}$  fructose samples run simultaneously with the 10 inulin samples of line 8 gave an average optical density  $\times 103$  of  $234 \pm 0.8$ , indicating complete hydrolysis of the inulin. The inulin densities in line 9 are somewhat lower than those usually obtained because some diphenylamine had precipitated after the reagent had stood 2 days in the refrigerator; simultaneous fructose determinations (not shown in table) were also correspondingly low.

60 ml of conc. HCl. This is the same reagent used by Harrison. Prepare fresh each day; if kept in a refrigerator some of the diphenylamine will precipitate and it is difficult to redissolve it. Use of this reagent after partial precipitation of diphenylamine yields a calibration curve which is lower than one obtained with fresh reagent.

**Procedure.** Pipette 2 ml of a 1:10 plasma filtrate<sup>7</sup> or appropriate urine dilution into a test tube 15  $\times$  125 mm. Add 4 ml of diphenylamine reagent and mix well with swirling. Since the reagent has a high viscosity, care must be taken that pipette drainage is adequate. For plasma determinations use as a blank 2 ml of a 1:10 filtrate of plasma obtained just prior to the injection of inulin; 2 ml of water is used for urine blanks. Place in a rack and heat in a water bath at 75°C for 60 minutes. The bath used had a thermoregulator sensitive to  $\pm 0.7^\circ\text{C}$  at 75°C; a mechanical stirrer insured uniform heating of all samples. At the end of the heating period place rack in an ice bath for 1 to 2 minutes. If the tubes are kept too long at the lower temperature there

may be some precipitation of diphenylamine. Read the samples in a photoelectric colorimeter with a 640 filter or a spectrophotometer at 640  $m\mu$ .

When 2 ml samples containing 10 to 60  $\mu\text{g}$  of fructose or inulin in water solution are used, optical density plotted against concentration yields a straight line. This corresponds to a range from 5 to 30 mg per 100 ml of plasma since 2 ml of a 1:10 filtrate are used. Separate calibration curves may not superimpose. Since the curves are straight lines, the inclusion of a standard with each set of determinations makes it possible to correct the values read from the calibration curve.

Table I shows the optical densities obtained with various concentrations of fructose, inulin and glucose. There is good agreement between samples of the same concentration in any one heating batch (represented on a horizontal line). Within the range of concentrations shown in Table I a given amount of fructose yields 64 times as much color as the same amount of glucose; this same ratio holds when fructose color development is compared with that of glucose in concentrations of 0.125 and 0.250 mg/ml (not shown in table). In our experience the plasma glucose level of fasted subjects does not depart more than 10 mg % from the blank value during

<sup>†</sup> Since this paper went to press we have been using 2.5 g of diphenylamine and have been keeping the tubes covered while they are in the water bath. This has prevented crystallization of diphenylamine.

<sup>7</sup> Somogyi, M., *J. Biol. Chem.*, 1930, **86**, 655.

a clearance experiment; the fluctuation is usually less than this. With a plasma inulin level of 15 mg % a 10 mg % departure from the blank would produce an error of one % in the inulin value determined.

A series of determinations on different solutions containing both glucose and fructose were run in the same heating batch with the corresponding separate glucose and fructose concentrations. The ratios of the observed additive optical densities to the calculated were 0.993, 0.993, 1.003, 1.007, 1.015, 0.994, 0.994, 0.985, 1.010, 1.006, 1.033, 1.006, 1.020 and 0.987.

*Summary.* A modification of Harrison's diphenylamine method for fructose or inulin determination is described, heating being carried out at 75°C for 60 minutes. With this procedure the ratio of fructose to glucose color production is 64, as compared with a ratio of 8 at 100°C for 30 minutes. Inulin hydrolysis is complete, the standard error of the method is less than 1 percent, the color is light stable and the low color production by glucose permits one to omit fermentation except in the presence of unusual glucose fluctuations.

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### False Positive Trichina Precipitin Reactions in Neoplastic Disease. (17430)

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(Introduced by C. P. Rhoads)

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Because of high fever, palpebral edema, painful inflammation of feet and hands, and eosinophilia of 5% in a child with atypical lymphosarcoma, a trichina precipitin test was performed. The reaction was strongly positive in a dilution of 1:1280, the highest titre routinely tested. The clinical signs rapidly subsided during treatment with 4-aminopteroylaspartic acid, a trichina complement-fixation test was negative, and x-ray examination demonstrated lesions of bone of the "moth-eaten" type commonly seen in acute leukemia. Hence, although the precipitin test remained unchanged, there was no longer any clinical reason to consider a diagnosis of trichiniasis and it was felt that the trichina precipitin reaction was a false positive.

This occurrence of an apparently false positive trichina precipitin test in a patient with lymphosarcoma led to a study of sera from other patients with lymphosarcoma, Hodgkin's disease, leukemia, and other neoplastic diseases. Positive reactions were so frequently seen that it seemed desirable to report the

phenomenon.

*Method.* Sera were obtained from hospitalized and clinic patients and personnel of Memorial Hospital. Three categories were studied: (1) those neoplastic diseases which involve principally the reticuloendothelial system (Hodgkin's disease, lymphosarcoma, the leukemias, and multiple myeloma), (2) widespread metastatic disease of other tissue, both carcinomas and sarcomas, (3) apparently healthy controls. Group 2 was included as controls who showed advanced and debilitating disease comparable to that seen in the first group.

Precipitin tests were performed by the standard technic of the Bureau of Laboratories of the New York City Department of Health. The antigen consisted of a saline extract of *Trichinella spiralis* larvae obtained from infected rat muscle by digestion with pepsin and hydrochloric acid. Undiluted serum was pipetted into small bore test tubes and overlaid with antigen in dilutions of 1:160, 1:320, 1:640, and 1:1280 on a basis of weight of dried larvae. A positive test was indicated by the formation, after two hours at room temperature, of an opaque ring. A

\* Fellow of the American Cancer Society, recommended by the Committee on Growth of the National Research Council.

TABLE I.

Results of 171 *Trichina* Precipitin Tests from 128 Persons With and Without Neoplastic Disease. Numerals indicate number of patients, not number of serum samples. Figures in parentheses indicate patient on whom skin tests were done (all negative), and those in brackets indicate patients on whom complement fixation tests were done (all negative).

Diagnosis	Total No. patients	Trichina precipitin titre					
		Neg.	160	320	640	1280	variable
Hodgkin's disease	13	1			4	8[1](1)	
Lymphosarcoma	14	5		2	2	4[2]	1
Acute leukemia	25	10			2	7(3)	6
Chronic leukemia	13	10		1	1	1	
Multiple myeloma	3	2			1		
Nieman-Pick's disease	1	1					
Osteogenic sarcoma	3				1(1)	1	1
Wilm's tumor	1				1		
Rhabdomyosarcoma	1	1					
Bronchogenic carcinoma	3				1	2	
Cancer of breast	8	4	2	1	1		
Cancer of prostate	5	3		2			
" " tongue	1	1					
" " uterus	2				1	1	
Normal healthy adults	26	26					
Normal healthy children	9	9					

titre of 320 is generally considered significant for diagnosis of trichiniasis.

*Results.* Tests have been done on 171 serum samples from 128 persons. Results are summarized in Table I.

All normal controls tested<sup>†</sup> (26 adults and 9 children) had negative reactions.

Of 13 patients with Hodgkin's disease, 12, in all stages of disease, had highly positive reactions. The negative reactor was a 10 year old boy; all others were adults. Of 14 patients with lymphosarcoma, 5 had negative reactions. These 5 were all adults in good condition. Seven (including 4 children) had positive reactions in a high titre and all of these patients had terminal and rapidly progressive disease. One case of lymphosarcoma in good condition and one in the terminal stage of the disease gave intermediate titres (320). Of 13 patients with chronic leukemia (7 myeloid, 5 lymphatic), only 3 had positive titres. Each of these was in the terminal phase of myeloid leukemia and showed a large percentage of blasts in marrow and peripheral blood. Results in 25 cases of acute leukemia were variable. Nine patients with acute leukemia had negative titres on all serum samples

tested, 8 had consistently positive titres, and 8 had variable titres on serial specimens. Those who consistently had high titres all died without remission. Most patients who had hematologic remissions (on treatment with antagonists of pteroylglutamic acid) had either consistently negative titres (5 patients), or on serial tests reverted from positive titres to negative (3 patients).

No generalization can be made concerning patients with other types of neoplastic disease. Of the 3 patients with bronchogenic carcinoma and 3 with osteogenic sarcoma who were tested, all gave high positive titres. Thirteen patients were tested who had metastatic carcinoma of breast or of prostate. Only one of these had a high titre and 7 were negative. It is noteworthy (in view of the positive titres found in bronchogenic and osteogenic neoplasms) that most of the patients with these 2 diagnoses had metastases to bones or lungs or both. Other diagnoses are represented by too few cases to merit discussion.

Three sera showing highly positive precipitin titres (2 patients with lymphosarcoma and one with Hodgkin's disease) were tested by John Bozicevich of the National Institutes of Health for complement-fixing antibodies with trichina antigen. All 3 were negative. Skin tests were performed with trichina antigen

<sup>†</sup> Twelve of the adult control sera were obtained by Dr. Joseph J. Bunim of The New York University Medical School.



diluted 1:8000 on 5 patients having strongly positive precipitin reactions. All were negative (Table I).

*Discussion.* The frequency of false-positive reactions in the presented data naturally raises questions as to the significance of these results, and the cause and mechanism of the reaction.

*Reliability of data.* All tests were performed by one person (A.E.T.) and were done without any knowledge of the diagnosis. The reproducibility of results in serial specimens from many of the patients (chiefly those with Hodgkin's disease, lymphosarcoma, and leukemias) indicate that the data as presented are reliable. Thirty-six patients had at least duplicate tests and only 10 have showed variation of more than one dilution. Those who showed variation in titre also showed concurrent variations in the disease process.

*Reliability of the method.* In established trichiniasis the precipitin test shows a fairly high degree of reliability. In recent acute trichiniasis most workers have reported over 90% positive reactions (reviews of literature by Gould,<sup>1</sup> and by Shookhoff *et al.*<sup>2</sup>).

False positive reactions are known to occur. Gould<sup>1</sup> reports 51 patients in whom trichina could not be demonstrated at autopsy and in whom precipitin tests had been performed. Ten of these (19.8%) had a positive reaction at a dilution of 1:100. The diagnoses of these patients are not reported. Bachman *et al.*<sup>3</sup> studied 857 sera from an area where trichiniasis is not endemic and found 8.4% apparently-false positive tests in patients with a wide variety of non-neoplastic disease. Augustine and Theiler<sup>4</sup> in a similar study found 18 apparently false-positive

reactions (12%) among 115 patients and felt that the false positives were related to medications (quinine, mercury, bismuth, and arsenic). There was no apparent relation to these same medications in Bachman's series. A single case of periarteritis nodosa reported by Reimann, Price, and Herbut<sup>5</sup> showed a false positive trichina precipitin test. Bassen, Thomson, and Silver<sup>6</sup> reported false positive precipitin tests in 5 cases of infectious mononucleosis.

*Significance of present data.* It is not probable that the reactions reported here indicate subclinical trichiniasis. No history suggestive of trichiniasis was elicited from any patient except the first patient tested. The available evidence indicates that the trichina precipitin test remains positive for only 2 to 3 years after infection, whereas skin reactions are more persistent.<sup>1</sup> In contrast to this, in the present study precipitin tests were positive, but skin tests and complement fixation tests were negative. Trichiniasis is rare in children,<sup>1</sup> while in the present study 14 of 21 children with leukemia or lymphosarcoma had high titres. Furthermore, if the present results were due to unrecognized trichiniasis an equal distribution of positive reactions would be expected in all diagnoses.

The major import, then, of the present study is that the trichina precipitin reaction is invalid as a test for trichiniasis in patients with certain neoplastic diseases, especially Hodgkin's disease, lymphosarcoma, and acute leukemia. The data also imply the possibility that this phenomenon might be utilized in prognosis, but the investigation has not yet proceeded far enough to permit valid discussion of such a possibility.

Indications at present suggest that the positive reactions are related in some way to the neoplastic process. There has been no correlation between positive precipitin reactions and fever or eosinophilia. The infrequency of positive reactions among patients with advanced carcinoma of the breast or prostate and the frequency of positive tests among

<sup>1</sup> Gould, S. E., Immunologic reactions in human helminthology, with special reference to trichinosis. (D.S. thesis, Wayne University College of Medicine), Edwards Brothers, Inc., Ann Arbor, Mich., 1942.

<sup>2</sup> Shookhoff, H. B., Birnkrant, W. B., and Greenberg, M., *Am. J. Public Health*, 1946, **36**, 1403.

<sup>3</sup> Bachman, G. W., Rodríguez-Molina, R., and Oliver-González, J., *Am. J. Hygiene*, 1934, **20**, 415.

<sup>4</sup> Augustine, D. L., and Theiler, H., *Parasitology*, 1932, **24**, 60.

<sup>5</sup> Reimann, H. A., Prier, A. H., and Herbut, P. A., *J.A.M.A.*, 1943, **122**, 274.

<sup>6</sup> Bassen, F. A., Thomson, A. E., and Silver, A., *J. Lab. and Clin. Med.*, 1949, **34**, 543.

patients in good condition with Hodgkin's disease, make it unlikely that the positive reactions reflect merely debilitation. There have been no changes in titres in serial specimens which would suggest that the presence in the serum of folic acid, folic antagonists, or nitrogen mustards cause the positive reactions. Many highly positive reactions were observed in sera obtained before any treatment had been given. The addition of folic acid (pteroylglutamic acid) or 4-amino-pteroylglutamic acid (10  $\gamma$ /cc) to serum having a negative precipitin reaction did not change the reaction.

*Mechanism of the reaction.* The cause of

these false positive trichina precipitin reactions is not known.

*Summary.* Patients with certain neoplastic diseases involving the reticuloendothelial system (Hodgkin's disease, lymphosarcoma and acute leukemia) exhibited a high percentage of false positive trichina precipitin reactions. Such reactions were much less frequently encountered in sera from patients with metastatic carcinomas and no positive reactions were observed in the normal controls. The false-positive reaction does not appear to be related to medication.

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## Tissue Distribution and Elimination of DDD and DDT Following Oral Administration to Dogs and Rats.\* (17431)

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Although the tissue distribution of ingested DDT (2,2-bis-(p-chlorophenyl)-1,1,1-trichloroethane) has been reported by several workers,<sup>1-7</sup> in most cases with particular reference to its storage in fat, similar data concerning its analog, DDD (2,2-bis-(p-chlorophenyl)-1,1-dichloroethane), is limited.<sup>5</sup>

The increasing agricultural use of DDD (Rhothane) against insect pests infesting fruit and vegetable crops makes desirable further knowledge concerning its tissue distribution

following ingestion. Although it has been demonstrated that DDD is less toxic on both an acute<sup>8,12</sup> and chronic<sup>5,11,12</sup> basis than is DDT, it has not as yet been made clear whether this difference is attributable to DDD being inherently a less toxic molecule, or whether it is due to differences in absorption, storage or excretion.

In an attempt to clarify this question of absorption, storage and excretion of DDD, the following experiments were done:

*Experimental. Dogs.* Ten female dogs were used, 5 receiving DDD and 5 DDT in a dose of 25 mg per kg daily except Sunday, for a maximum period of 4 weeks. The insecticides were administered orally once daily in the form of a 10% solution in corn oil in gelatin capsules at the time of the daily feeding.

<sup>8</sup> von Oettingen, W. F., and Sharpless, N. E., *J. Pharm. Exp. Therap.*, 1946, **88**, 400.

<sup>11</sup> Haag, H. B., Finnegan, J. K., Larson, P. S., Dreyfuss, M. L., Main, R. J., and Riese, W., *Ind. Med.*, 1948, **17**, 477.

<sup>12</sup> Smith, M. I., Bauer, H., Stohlman, E. F., and Lillie, R. D., *J. Pharm. Exp. Therap.*, 1946, **88**, 359.

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<sup>1</sup> Laug, E. P., and Fitzhugh, O. G., *J. Pharm. Exp. Therap.*, 1946, **87**, 18.

<sup>2</sup> Kunze, F., Nelson, A. A., Fitzhugh, O. G., and Laug, E. P., *Fed. Proc.*, 1949, **8**, 311.

<sup>3</sup> Woodard, G., Ofner, R. R., and Montgomery, C. M., *Science*, 1945, **102**, 177.

<sup>4</sup> Telford, H. S., and Guthrie, J. E., *Science*, 1945, **102**, 647.

<sup>5</sup> Woodard, G., Davidow, B., and Nelson, A. A., *Fed. Proc.*, 1948, **7**, 266.

<sup>6</sup> Smith, M. I., and Stohlman, E. F., *Pub. Health Rep.*, 1944, **59**, 984.

<sup>7</sup> Stohlman, E. F., and Smith, M. I., *J. Pharm. Exp. Therap.*, 1945, **84**, 375.

TABLE I.

DDD and DDT Content of Tissues of Dogs to Which the Insecticides Had Been Administered Orally Daily for Periods of 2 and 4 Weeks.

Insecticide	Insecticide content, mg per kg of tissue									
	DDD					DDT				
	2 wks		4 wks			2 wks		4 wks		
	D-4	D-5	D-1	D-2	D-3	T-4	T-5	T-1	T-2	T-3
Liver	0	0	1.6	0	24	0	0	0	0	0
Kidney	15	4.3	13	14	15	4.9	0	14	7.2	9.6
Heart	3.8	0	11	12	13	0	3.4	4.9	8.1	3.4
Brain	0	0	3.5	4.1	5.2	1.3	0	2.5	3.9	1.2
Lung	0	0	1.9	3.9	lost	0	0	0	0	0.8
Pancreas	0	0	8.9	20	12	0	3.4	14	9.4	8.8
Spleen	0	1.7	0	12	4.7	0	0	2.2	3.6	0
Adrenal	0	0	150	0	210	0	0	83	63	62
Fat	76	270	880	360	300	29	100	910	400	200
Muscle (gastroc.)	7.6	5.2	lost	20	28	8.4	5.5	12	14	18
Skin	90	7.2	28	128	18	82	73	0	6.4	3.3
Mammary gland	—	—	—	—	—	—	—	2.2	—	21
Fecal excretion mg/kg body wt, 48 hr	0.9	4.2	3.9	2.2	3.9	lost	0.03	0.01	0.5	0.8

Twenty-four hour urine samples were taken at the end of the first, second, and fourth weeks for subsequent analysis for DDD, DDT, or their acid end-product, DDA (2,2-bis-(p-chlorophenyl) acetic acid). The total feces of each dog was collected over the 48 hour period just prior to the time of sacrifice for similar chemical analysis.

At the end of 2 weeks, 2 of the dogs in each insecticide group were sacrificed, the remaining dogs being sacrificed at the end of 4 weeks. Samples of the tissues listed in Table I were removed, frozen quickly in a dry-ice-alcohol mixture and preserved in the frozen state for subsequent analysis for DDD and DDT. In the case of 2 dogs receiving DDT that were lactating at the end of the experiment a sample of mammary gland tissue was similarly prepared. Because the presence of blood in the tissue sample appears to interfere with the analytical procedure used, as much as possible was removed (ca. 90% as determined by hematocrit) from the circulation by saline perfusion before death.

During the course of the experiment, 3 of the dogs bore litters. One of the 4-week DDD dogs (D-1) bore 5 premature stillborn pups 13 days after beginning of the experiment. All of these were frozen whole for subsequent

TABLE II.

DDT Content of Pooled Organs from Pups Nursing from Mothers Receiving Daily Doses of DDT Orally.

Tissue	DDT, mg per kg organ weight	
	4 pups from T-3 (2 days old)	3 pups from T-1 (2 wks old)
Liver	220	0.51
Kidney	6.3	8.3
Heart	0	10
Lung	3.5	1.3
Brain	0	0
Skin	26	12
Fat	205	0
Pancreas	149	46
Spleen	9.8	0
Muscle	8.4	5.8

chemical analysis. On the same day, one of the 4-week DDT animals (T-1) bore 5 pups, 2 of which were sacrificed as soon after birth as they were discovered and frozen whole. The other 3 were sacrificed at the end of the experiment and the organs listed in Table II removed, pooled as to organ and frozen. Another 4-week DDT dog (T-3) bore 6 pups 2 days before the termination of the experiment. Two were killed and frozen whole before having an opportunity to nurse; the other 4 were killed at the end of the experiment and the same organs removed, pooled and frozen. Because of their small size, no

attempt was made to perfuse out the blood of the pups.

*Rats.* The rats used in this experiment were survivors from a series of animals that had been subjected to a 2-year feeding program comparing the chronic toxicity of DDD with that of DDT. Finely ground Purina Dog Chow served as the basic diet, and into this was incorporated predetermined amounts of 5 or 10% DDD or DDT in Pyrax<sup>†</sup> to achieve the dietary concentrations listed in Table III. Only analyses of the fat were done on these animals.

All DDD, DDT and DDA determinations were done according to the Schechter-Haller method.<sup>9,10</sup>

*Results and Discussion. Dogs.* Table I shows the amounts of DDD and DDT in mg per kg of organ weight found in the tissues of the treated animals.

It will be noted that in the animals sacrificed at the end of 2 weeks the fat and the skin were the main sites of storage of both DDD and DDT. Muscle and kidney showed a measurable level of both insecticides, however, the other tissues that were studied showed either none or very small amounts.

In those animals sacrificed at the end of 4 weeks the fat, again, showed the highest content of both DDD and DDT, whereas, the skin content, which was relatively high for both insecticides at the end of 2 weeks, was found to be low in the case of the DDT animals. This lower skin content of DDT may be related to the body weight loss and resultant probable decrease in cutaneous fat content incurred by this group.

It seems that, in general DDD and DDT were deposited in fat to a similar degree. This is in agreement with the thesis of von Oettingen and Sharpless<sup>6</sup> that since the 2 compounds have similar solubilities in olive oil they would probably be stored to about the same degree in fat.

The other tissues in which little or no insecticide was found at the end of the 2 week

period contained definite quantities of DDD or DDT at the end of 4 weeks. This is particularly noticeable in the case of the adrenal gland and is most apparent here in those animals which received DDD. This latter observation is of interest in relation to the adrenal cortical atrophy that has been described in dogs receiving DDD orally<sup>5,13</sup> and which has also been noted in this laboratory (unpublished).

Analysis of the pooled carcasses of the 5 stillborn pups from one of the 4-week DDD dogs (D-1) showed a content of 3.7 mg per kg DDD. Similar analyses of individual carcasses of the 2 newborn pups from dog T-1 and the 2 newborn pups from dog T-3 (both 4-week DDT dogs) gave average values of 1.3 mg per kg for the first pair and 0.04 mg per kg for the second. It was thought that the latter result was low owing to possible loss of part of the sample. These results demonstrate transfer of both DDD and DDT across the placenta.

Table II shows the amounts of DDT stored in the pooled organs of pups that were born to 2 of the dogs (T-1 and T-3) during the feeding period and nursed for the remainder of the time until the end of the experiment. It will be noted that the pups from dog T-3, which were 2 days old at the time of sacrifice, showed high levels of DDT in the liver, skin, fat and pancreas, whereas, those that had nursed for a period of 2 weeks (pups from dog T-1) showed much lower levels in these tissues, and, with the exception of heart tissue and perhaps kidney, showed lower levels in all other organs examined. This again seems indicative of a high degree of placental transfer of DDT and further suggests that the fetus is more liable to the accumulation of DDT than is the suckling offspring, despite the fact that DDT has been shown to be secreted in the milk.<sup>2,4</sup>

Included in Table I are data showing the amounts of the insecticides excreted in the feces in the 48 hour period just prior to sacrifice. Fecal excretion, in all cases, represented but a small proportion of the amount of the

<sup>†</sup> A pyrophyllite diluent.

<sup>9</sup> Ofner, R. R., and Calvery, H. O., *J. Pharm. Exp. Therap.*, 1945, **85**, 363.

<sup>10</sup> Schechter, M. S., and Haller, H. L., *J. Am. Chem. Soc.*, 1944, **66**, 2129.

<sup>13</sup> Nelson, A. A., and Woodard, G., *Fed. Proc.*, 1948, **7**, 276.

TABLE III.  
Averages of Fat Analyses for DDD and DDT in Rats at the End of Two Year Feeding.

Insecticide	Dietary concentration parts per million	No. of rats	Content of DDD or DDT in body fat, mg/kg (Avg and range)
DDD	150	3	220 (180-250)
	300	7	270 (140-830)
	600	4	480 (110-950)
	900	4	350 (280-500)
	1,200	10	420 ( 60-790)
	1,800	5	310 (210-430)
	2,500	5	680 (320-940)
	3,750	1	1,290
DDT	150	3	2,070 (400-5,400)
	300	5	470 (240-690)
	600	1	340

insecticide administered during that period. It is apparent that DDD is uniformly excreted to a greater degree in the feces than is DDT.

In contrast to the findings of Smith *et al.*<sup>6,12</sup> that orally administered DDT is excreted in the urine of rabbits, it does not appear to be excreted in the urine of dogs. Neither does the dog appear to excrete DDD by the way of the kidney. However, DDA appears to be a urinary end-product common to both insecticides in dogs. Thus the average 24 hour excretion of DDD as DDA was 0.63 mg per kg body weight at the end of the first week of administration, 0.50 mg at the end of the second week, and 1.25 mg at the end of the fourth week. In the case of DDT, the DDA excretion figures were 0.13 mg, 0.12 mg, and 0.17 mg per kg body weight at the end of the same intervals. These figures also indicate that the rate of DDD excretion in the form of DDA is greater than in the case of DDT.

*Rats.* It is apparent from Table III that both DDD and DDT are stored in considerable amount in the fatty tissues of the rat. Among the DDD-treated animals there appears to be a tendency for deposition of DDD to increase with its increasing dietary

content, although this is not entirely consistent. The number of animals and dietary levels studied using DDT was too limited to permit similar comparisons.

*Conclusions.* 1. DDD and DDT when administered orally are stored in the tissues of the dog, the greatest storage appearing in the fat.

2. Similarly DDD and DDT are both stored in the fat of the rat.

3. Both DDD and DDT are transported across the placental barrier in the dog.

4. In the dog, a small proportion of orally administered DDD or DDT is excreted in the feces. Of the 2, DDD appears in greater amount.

5. When administered orally to the dog, neither DDD nor DDT are excreted as such in the urine. DDA is a urinary end-product common to both DDD and DDT, appearing in greater amount following DDD administration.

We wish to extend our thanks to Dr. E. L. Stanley of the Rohm and Haas Company for performing the analytical procedures connected with this study.

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## Excretion of Coproporphyrin in Rats Developing Acute Massive Hepatic Necrosis.\* (17432)

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Massive hepatic necrosis in rats maintained on diets low in sulfur containing amino acids has been well described by Himsworth and Glynn.<sup>1</sup> This is a rapidly fatal disease which appears suddenly in a hitherto healthy animal. The lesion has been produced by feeding diets deficient in cystine and methionine where all of the nitrogen was supplied by other amino acids.<sup>2</sup> Hock and Fink<sup>3</sup> and Himsworth and Glynn<sup>1</sup> regularly observed hepatic necrosis on diets containing yeast as a sole source of protein. Recently Gyorgy<sup>4</sup> has described the regular production of this lesion when a certain type of yeast was employed as the protein source. The suddenness with which illness and death appear in such animals has been well documented<sup>5,6</sup> and no satisfactory biochemical means of predicting the onset of this lesion have been described. The present study was undertaken to determine whether or not an increase in the urinary excretion of coproporphyrin might herald the development of massive hepatic necrosis.

The urinary excretion of coproporphyrin in liver disease has been discussed in recent reviews.<sup>7,8</sup> An increase of urinary coproporphyrin is frequently regarded as a sensitive

index of liver functional impairment. Clinical applications have been recently described by Watson and his associates<sup>9</sup> with particular reference to the distribution of the type I and type III isomer.

Glynn, Himsworth and Neuberger<sup>2</sup> stated that in the course of their studies on massive hepatic necrosis, the urines from certain groups of rats had been examined for porphyrins. No significant increases were found; the actual values obtained were not presented. Dent and Rimington,<sup>6</sup> during their attempts to produce acute hepatic necrosis, observed the rapid development of porphyrinuria in rats fed a diet which included oxidized casein. Although the diets were devoid of methionine and contained very little cystine, the rats failed to develop acute necrosis of the liver. This study, however, left unanswered the question raised above, viz: whether a heightened excretion of coproporphyrin might precede the manifestations of massive liver necrosis.

**Methods.** The diet employed in this experiment was identical with those employed by Gyorgy.<sup>4†</sup> The composition of the diet was powdered yeast,<sup>‡</sup> 18%, cornstarch, 79% and salt mixture, 3%. Each animal received 8 g of the mixture per day. Each daily portion was supplemented with 0.5 cc of peanut oil and 2 drops of cod liver oil. Daily, each rat received 1.0 cc of a solution containing thiamine chloride 20  $\gamma$ , calcium pantothenate 100  $\gamma$ , pyridoxine 20  $\gamma$  and riboflavin 25  $\gamma$ .

The rats were maintained in individual cages. Weights were recorded once each week.

\* This investigation was supported in part by the Commission on Liver Disease, of the Army Epidemiological Board, Office of the Surgeon General, Washington, D.C.

<sup>1</sup> Himsworth, H. P., and Glynn, L. E., *Clin. Science*, 1944, **5**, 93.

<sup>2</sup> Glynn, L. E., Himsworth, H. P., and Neuberger, A., *Brit. J. Exp. Path.*, 1945, **26**, 326.

<sup>3</sup> Hock, A., and Fink, H., *Z. Physiol. Chem.*, 1943, **274**, 187.

<sup>4</sup> Gyorgy, Paul, Liver Injury, Transactions of the Eighth Conference, Josiah Macy Jr., Foundation, New York, 1949, in press.

<sup>5</sup> Himsworth, H. P., *The Liver and Its Diseases*, Oxford, 1947. Blackwell Scientific Publications.

<sup>6</sup> Dent, C. E., and Rimington, C., *Biochem. J.*, 1947, **41**, 253.

<sup>7</sup> Dobriner, K., and Rhoads, C. P., *Physiol. Rev.*, 1940, **20**, 416.

<sup>8</sup> Watson, C. J., and Larson, E. A., *Physiol. Rev.*, 1947, **27**, 478.

<sup>9</sup> Watson, C. J., Hawkinson, V., Capps, R. B., and Rappaport, E. M., *J. Clin. Invest.*, 1949, **28**, 621.

† The initial supply of yeast employed in these studies was made available to us by Dr. Gyorgy.

‡ "Kiln dried baking yeast," United Yeast Co., Ltd., 241 City Road, London E.C.1.

Food was supplied, in weighed amounts, every 2 days and water was allowed freely. Urine and feces collections were made for 48 hour periods at various intervals during the course of the study. During the collection period the rats were housed in individual plastic cages with glass rod bottoms. These cages were suspended over large glass funnels with 5 cm openings at the tapered end. Satisfactory separation of urine and feces, with little or no contamination, was accomplished by means of a specially devised glass tube attached to the lower end of the funnel. This successfully collected the urine at the side, where it drained into a tube, and permitted the feces to drop straight through into a separate container. The precaution of employing only glass and plastic in these devices was deemed necessary to prevent any contact of the urine with metal. We have noted the formation of metal porphyrin complexes during other studies when metal cages were employed, as have others also.<sup>6</sup> The collection periods were interrupted for 60 minutes at the end of the first 24 hours to permit feeding. An average of 3 to 5 cc of clear urine was obtained during each 24 hour period from rats weighing 40 to 90 g each. The sides of the funnels were washed down with 10 cc of water at the end of each collection period. The urine plus the washings were then refrigerated. The coproporphyrin analyses were carried out on the total 48 hour urine and fecal collections within 12 hours of the final collection time.

The estimation of the fecal coproporphyrin was conducted according to the method of Schwartz and Zagaria.<sup>10</sup> This method was slightly modified to permit its use in the analyses of the urine samples. During the analyses of both urine and feces, the initial 5% HCl samples were each diluted to 0.4% and extracted exhaustively with chloroform to remove protoporphyrin and deuteroporphyrin. The completeness of the separation of the coproporphyrin from these two porphyrins was checked by examining the chloroform washings under the ultra-violet light.

The coproporphyrin was quantitated by readings in the fluorophotometer (Lumetron) using a solution of 1  $\mu$ g of coproporphyrin per 1 cc as the standard reference.

Estimation of the coproporphyrin isomer content was performed by pooling the 1% HCl fractions from 3 urine or 3 fecal collections. Procedure B of the method described by Schwartz and his co-workers<sup>11</sup> was employed for the determination of the percentage of isomer I and III.

**Results.** Experimental observations were made on 21 rats whose initial weights ranged from 36 to 88 g. The data obtained is summarized in Table I. Fourteen of the rats died spontaneously between the 30th and 125th day. Each of these animals exhibited the characteristic gross and microscopic findings of acute massive hepatic necrosis as described and illustrated by Himsworth.<sup>5</sup> Representative specimens are shown in Fig. 1 and 2. In most instances the rats were found dead; in a few instances the abrupt change from an apparently normal healthy status to a moribund one was observed in a matter of a few hours. Occasionally convulsions preceded death. No attempt was made to administer glucose solution, though hypoglycemia has not been excluded as a possible cause of the terminal convulsive seizures.

The remaining 7 animals were sacrificed after 41, 51 and 59 days as shown in Table I. In no instance in this group was it possible to detect gross or microscopic changes in the liver.

The data relating to the urinary and fecal coproporphyrin excretion is shown in Table I. Because of other experiments it was not possible to house the animals in the special collecting chambers all of the time; random samplings were therefore obtained. Control values for 48 hour collections of urine and feces in 6 rats maintained on a diet of fox chow just before the start of this experiment are shown in Table I. An additional 6 rats of similar weight and maintained on a similar control diet revealed urinary values of 4 to 20  $\gamma$  of coproporphyrin and fecal values of

<sup>10</sup> Schwartz, S., and Zagaria, R., Metallurgical Laboratory Report CH-3600, Chicago, 1946.

<sup>11</sup> Schwartz, S., Hawkinson, V., Cohen, S., and Watson, C. J., *J.B.C.*, 1947, **168**, 133.

TABLE I. Urine and Fecal Coproporphyrin Excretion in  $\mu\text{g}$  per 48 hr in Rats Maintained on an 18% Yeast Diet.

Rat No.	Sex	Material studied	Control values	Day of experiment								Day terminated	Initial wt (g)	Final wt (g)	Outcome	Hist. find. (liver)
				11	19	23	31	35	39	42	44	49				
52	F	U		7	5							30	40	42	D	Massive necrosis
55	M	U			8		14					38	42	60	D	"
53	M	U			3		5					41	36	48	D	"
54	M	U			15		17					41	36	53	D	"
56	M	U			4		7					41	36	57	D	"
43	M	U			4		12					41	70	78	D	"
63	M	U	16	22	9	11	2	19	6*			44	73	71	D	"
44	M	U	9					3	19	8		58	58	82	D	"
41	M	U						25	3		9	6	66	80	D	"
60	M	U	16					5	13			59	71	86	D	"
65	F	U	11					13	5			59	60	75	D	"
40	M	U	15					5				59	60	88	D	"
45	M	U	5	21	6	19	8	4	4	12	15	62	63	86	D	"
42	M	U						20	5	7	6	74	74	90	D	"
50	M	U	18					5				125	78	88	D	"
51	M	U	9					18	9	13	17	79	88	90	D	"
57	M	U	10									41	40	65	S	Normal
58	M	U	8	18	13							41	40	44	S	"
59	M	U		10	9							51	65	69	S	"
64	M	U		34	4	19	5	20	17	13	3	51	60	58	S	"
61	M	U		18	11	18	4	15	15	6		51	63	66	S	"
				22	23	4	4	10	13	5		59	62	74	S	"
				5	4			14	23	3	4	81	60	69	S	"
				24	8	2	2	19	3	2						

U—Urine.

F—Feces.

D—Spontaneous death.

S—Sacrificed.

\* 24 hr collection; animal died during second 24 hr period.

† Animals maintained on stock diet (Fox Chow).

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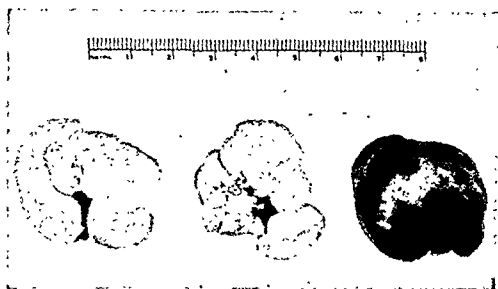


FIG. 1.

Gross appearance of rat liver showing massive hepatic necrosis as contrasted with unaffected experimental animal and a normal control, photographed after 4 hours fixation in formalin. The dark areas in the right hand specimen represent extensive areas of hemorrhagic necrosis (rat 56, death on 41st day). The center specimen (rat 51, sacrificed on 41st day) revealed no evidence of necrosis. The specimen to the left was taken from a normal animal of similar weight.

6 to 13  $\gamma$  for 48 hour periods.

It is apparent from these data that no significant increases in the urinary excretion of coproporphyrin occurred in these animals. Rat 56 died suddenly during the second 24 hours of the collection period. The urinary and fecal values obtained during the first period (Table I) were not elevated. Estimation of the coproporphyrin isomer content was

made in pooled samples of the material under study. Eighty to 90% of the coproporphyrin present behaved as the type III isomer by the differential precipitation "fluorescence quenching" technic.<sup>11</sup> A similar finding was noted in urine and fecal samples from the normal rats.

As has also been noted by others,<sup>2,6</sup> a few of the animals in this study exhibited traces of bilirubin in the urine one or two days before death but this was not common or constant. No increases in urobilinogen were observed; urinary amino acid excretion was not studied. A few specimens of urine were examined for the presence of homogentisic acid with negative results. The presence of this or a closely related substance in the urine of rats developing hepatic necrosis was observed by Glynn, Himsworth and Neuberger.<sup>2</sup> This occurred in rats fed an amino acid diet deficient in cystine and methionine. The question as to whether or not renal damage might have prevented the excretion of pathological amounts of coproporphyrin may be raised. Histologically the kidneys of these animals appeared normal.

The excretion of excessive amounts of coproporphyrin III in the urine of humans is a



FIG. 2.

Photomicrograph, liver, rat 56, areas of necrosis in contrast with normal appearing liver cells, are shown in this section. In most sections, the areas of necrosis are more extensive. (H and E  $\times 125$ ).

frequent accompaniment of chemical or heavy metal toxicity, with or without evidence of hepatic functional impairment.<sup>8,9</sup> The failure to observe excesses in this experiment is in better agreement with the belief that the acute massive hepatic necrosis is not due to a chemical or poison. The histologic appearance of these livers suggests that the lesion develops very rapidly since there appears to be little evidence of antecedent damage as judged by infiltration or any attempt at a reparative process.

**Summary.** 1. The urinary and fecal excretion of coproporphyrin was studied at varying intervals in 21 young rats maintained on a

diet containing 18% yeast as a source of protein.

2. Fourteen of the rats died suddenly between the 30th and the 125th day of acute massive hepatic necrosis. The gross and microscopic findings were identical with those described by Himsworth as characteristic of this lesion.

3. No significant increase in the excretion of coproporphyrin was observed during the course of these experiments. This is believed to minimize the likelihood of chemical or metal toxicity as the cause of the necrosis.

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### Comparative Effect of Aureomycin and Chloromycetin on Psittacosis Infection in Chick Embryos.\* (17433)

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Aureomycin<sup>1</sup> and chloromycetin<sup>2,3</sup> are antibiotics which have a similar spectrum of activity that includes many bacteria, rickettsias and viruses of the psittacosis-lymphogranuloma venereum group. With respect to psittacosis, Wong and Cox<sup>4</sup> found aureomycin to be highly effective against infection of embryonated hen's eggs with the 6BC strain, and Smadel and Jackson,<sup>5</sup> using the same strain, demonstrated similar beneficial effects from chloromycetin. The conditions of the experiments of these 2 groups of workers, however, were not entirely comparable. It seemed of

interest, therefore, to make a direct comparison of the effects of these 2 agents under more nearly identical conditions. Such a comparison is reported in this paper.

**Materials and methods.** A uniform source of virus for the present experiments was obtained in the following manner: A stock yolk-sac suspension of the 6BC strain of psittacosis virus was injected into the yolk sac of a number of 7-day chick embryos which were then incubated at 35°C. On the 5th day, when deaths began to occur, the yolk sacs were harvested and pooled. An equal volume of broth was added and the mixture homogenized in a Waring blender. The resulting 50% yolk sac suspension which, on culture, was found to be free of bacterial contamination, was distributed in sealed pyrex ampoules, quick-frozen and stored at -70°C. The LD<sub>50</sub> of this suspension was about 10<sup>-5</sup> throughout these experiments. Yolk sac smears of infected embryos stained by Macchiavello's technic showed numerous characteristic elementary bodies.

A fresh solution of crystalline aureomycin

\* Aided by a grant from the United States Public Health Service.

<sup>†</sup> Research Fellow in Virus Diseases, National Research Council.

<sup>1</sup> Duggar, B. M., and others, *Ann. N. Y. Acad. Sci.*, 1948, **51**, 175.

<sup>2</sup> Gottlieb, D., Bhattacharyya, P. K., Anderson, H. W., and Carter, H. E., *J. Bact.*, 1948, **55**, 409.

<sup>3</sup> Smith, R. M., et al., *J. Bact.*, 1948, **55**, 425.

<sup>4</sup> Wong, S. C., and Cox, H. R., *Ann. N. Y. Acad. Sci.*, 1948, **51**, 290.

<sup>5</sup> Smadel, J. E., and Jackson, E. B., *Science*, 1947, **100**, 418.

hydrochloride<sup>†</sup> was prepared just prior to use in each experiment. A solution containing 8 mg of crystalline chloroamphenicol<sup>‡</sup> (chloromycetin) per ml was made, also in broth, but this was heated to 100°C for 15 minutes and then stored at 4°C for use in 2 or 3 experiments. The chloromycetin remained in solution throughout this study and retained its potency when assayed against a standard test organism. Both antibiotics were diluted for use so that the desired dose was contained in 0.25 ml of broth.

All inoculations were made into the yolk sac of 7-day-old embryonated eggs. In each experiment, groups of 6 to 12 eggs per dose were each injected with aureomycin in amounts ranging from 1.0 mg to 0.0625 mg and comparable groups were inoculated with similar amounts of chloromycetin. These eggs were then inoculated with a 10<sup>-3</sup> dilution of the virus suspension. A titration of the virus was included in each experiment and this inoculum was regularly found to contain about 10<sup>5</sup>LD<sub>50</sub>. The time elapsed between injections of drug and virus was about 45 minutes. Uninfected drug controls and untreated virus-infected controls were each given a second injection of plain broth. The eggs were incubated at 35°C; they were candled and the deaths noted daily for 12 days, after which the experiment was terminated. Deaths occurring before the third day were considered traumatic. The dose of drugs that protected 50% of embryos (PD<sub>50</sub>), and the LD<sub>50</sub> were calculated by the method of Reed and Muench.<sup>6</sup> This method was also used to calculate the day when 50% of the embryos died (DD<sub>50</sub>).<sup>||</sup>

**Results.** Similar results were obtained in 6 separate experiments. The data for all 6 experiments have been combined in Table I.

<sup>†</sup> Supplied in sterile vials by the Lederle Laboratories.

<sup>‡</sup> Furnished by the Department of Clinical Investigation, Parke, Davis and Co.

<sup>6</sup> Reed, L. J., and Muench, H., *Am. J. Hyg.*, 1938, **27**, 493.

<sup>||</sup> This method was chosen in preference to the usual calculation for "mean day of death" in order to avoid the error introduced by the embryos which survived at the end of the experiment.

TABLE I. Effect of Aureomycin and Chloromycetin on Psittacosis (GBC) Infection of Chick Embryos. Summation of 6 experiments.

Antibiotic	Dose, mg	No. of embryos infected*	No. of embryos surviving												% survivors			
			Days after infection															
			3	4	5	6	7	8	9	10	11	12	DD <sub>50</sub> †	MPL <sub>50</sub> ‡		Days after infection 8 10 12		
Aureomycin	1.0	22	22	21	21	20	19	19	15	13	12	10	11.8	7.2	86	59	45	
	.5	54	54	53	52	50	47	38	37	30	27	21	11.6	7.0	70	56	39	
	.25	56	56	55	54	54	51	37	24	15	9	6	9.7	5.1	66	27	11	
	.125	57	57	56	55	46	41	22	10	5	0		8.1	3.5	39	9	0	
	.0625	35	35	35	33	23	15	5	1	0			7.0	2.4	14	0	0	
Chloromycetin	1.0	43	43	42	39	36	34	24	19	11	6	3	9.5	4.9	56	26	7	
	.5	50	50	50	49	48	37	26	10	3	0		8.3	3.7	52	6	0	
	.25	53	53	52	47	37	17	3	1	0			6.7	2.1	6	0	0	
	.125	52	52	52	44	21	2	0					5.9	1.3	0	0	0	
	.0625	12	12	11	7	0							5.1	0.5	0	0	0	
Broth		57	57	51	10	0							4.6		0	0	0	
Infecting dose per embryo = 85,000 I.D. calculated as 0.1 ml.																		

\* Number which survived more than 2 days.

† Day when 50% of embryos died (calculated).

‡ Mean prolongation of life. (DD<sub>50</sub> for each dose minus DD<sub>50</sub> for unprotected controls).

(See also Table II).

Infected dose per embryo = 85,000 LD<sub>50</sub>, calculated on 8th day.

TABLE II.  
Protective Dose.

Days after infection	PD <sub>50</sub> (mg)		LD <sub>50</sub> of infecting dose*
	Aureomycin	Chloromycetin	
7	.09	0.36	$1.8 \times 10^4$
8	.20	0.61	$8.5 \times 10^4$
9	.31	>1	$3.1 \times 10^5$
10	.42	>1	$6.2 \times 10^5$
11	.51	>1	$9.6 \times 10^5$
12	.62	>1	>10 <sup>6</sup>

\* The number of LD<sub>50</sub> contained in the infecting dose will vary according to the time of observation; and the figures in this column indicate the apparent number of LD<sub>50</sub> on the days indicated in the first column.

The infected and unprotected control embryos all died between the 4th and 6th days. The DD<sub>50</sub> for this group was 4.6 days. In the treated groups, the DD<sub>50</sub> varied directly with the dose.

The beneficial effects of the 2 antibiotics may be compared on the basis of the mean prolongation of life (MPL) which, for convenience may be considered as the difference between the DD<sub>50</sub> of any treated group and that of the control group. The MPL for the aureomycin-treated eggs was greater than that of the chloromycetin-treated groups at each dosage level. The MPL for the groups of embryos receiving doses of 0.5 and 0.25 mg of aureomycin was 3.3 and 3.0 days longer, respectively, than for those receiving the corresponding doses of chloromycetin. At each of the other dosage levels the MPL was about 2 days longer in the aureomycin treated groups.

The greater protective effect of aureomycin as compared with chloromycetin is also strikingly apparent from the percent of survivors among the groups of embryos treated with various doses of antibiotics. This was calculated for each dose at different times after infection and the figures for the 8th, 10th and 12th days are shown in Table I.

A more quantitative estimation of the relative effectiveness of the 2 drugs may be arrived at by comparing the dose required to protect 50% of the embryos (PD<sub>50</sub>) for any given number of days after infection. The result is shown in Table II for days 7 to 12, inclusive. In the case of aureomycin, it is seen that the PD<sub>50</sub> increased at a steady rate of approximately 0.1 mg per day during this period. The increase for chloromycetin was

at a much more rapid rate between the 7th and 9th days, after which time the PD<sub>50</sub> of this agent exceeded 1.0 mg, the largest dose used in these experiments. The PD<sub>50</sub> of aureomycin on days 7, 8 and 9 was 0.09, 0.20 and 0.31 mg, respectively, as compared with 0.36, 0.61 and more than 1.0 mg for chloromycetin on the corresponding days. Thus on these 3 days, aureomycin was from 3 to 4 times as active as chloromycetin, weight for weight, in protecting the embryos against the infecting dose of virus used. A similar advantage in favor of aureomycin was noted after the 9th day, but this was not quantitated since the PD<sub>50</sub> for chloromycetin was greater than 1.0 mg, the largest dose used. [The infecting dose on the basis of the LD<sub>50</sub> calculated for each of these days, is also shown in Table II.]

**Discussion.** The results indicate that the life of chick embryos infected with the 6BC strain of psittacosis virus was prolonged for an average of from 2 to more than 3 days longer by aureomycin than they were by the same dose of chloromycetin. Also, the dose of chloromycetin required to protect 50% of infected embryos for 7 to 9 days was 3 to 4 times as great as the dose of aureomycin required to produce the same effect. Since the molecular weight of aureomycin is 508<sup>7</sup> as compared with 323 for chloromycetin,<sup>8</sup> the PD<sub>50</sub> of the former is more than 5 times that of the latter on a molecular basis.

Statistically, the observed differences were highly significant.

<sup>7</sup> Broschard, R. W., *et al.*, *Science*, 1949, **109**, 199.

<sup>8</sup> Controulis, J., Rebstock, M. C., and Crooks, H. M., Jr., *J. Am. Chem. Soc.*, 1949, in press.

These findings were rather unexpected in view of the rapid deterioration of aureomycin *in vitro* at neutral or alkaline pH, and at the temperature employed.<sup>9</sup> Other factors in the embryo, however may affect the stability of the two agents and some of these factors are now under study.

**Conclusions.** Both aureomycin and chloromycetin prolong the life of chick embryos infected with the 6BC strain of psittacosis virus. There is a direct relation between the dose of antibiotic and the prolongation of life of

<sup>9</sup> Paine, T. F., Jr., Collins, H. S., and Finland, M., *J. Bact.*, 1948, **56**, 489.

the embryos, the infecting dose being kept constant. With equal doses of the antibiotics, aureomycin prolonged the life of the embryos for an average of from 2 to more than 3 days longer than chloromycetin, depending on the dose used.

On a weight basis, aureomycin was more than 3 times as effective in protecting chick embryos against the dose of virus that was used in this study. On a molecular basis the aureomycin was more than 5 times as effective as chloromycetin in this infection.

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### Hypotension Associated with Nutritive Failure.\* (17434)

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During the 12 years we have been studying persons with nutritive failure in a large Nutrition Clinic, we have learned that there is a great variation in blood pressure among such persons. Readings range from those which are too high to be obtained on an ordinary sphygmomanometer to those which are extremely low. In general, however, we have found that in the person with nutritive failure the blood pressure usually is below normal and that it tends to rise slowly as the nutritive failure is corrected. Even in patients with both hypertension and nutritive failure, we have observed that the blood pressure tends to increase slightly when the nutritive failure is corrected and that they lead a more energetic life.

It occurred to us that it would be worthwhile to determine the effect of desoxycorticosterone acetate on blood pressure. Since it has been known for some time that the development of edema, hypertension, and cardiac

failure are the chief dangers associated with overdosage of desoxycorticosterone acetate,<sup>1-4</sup> we selected for study 3 persons who had been under observation in our Clinic for many years and who had every evidence of a properly functioning cardiovascular system except that the blood pressure was below normal. Each morning for a month these patients came to the Clinic early for special consideration and study. After they became accustomed to this routine, their blood pressures were taken under resting conditions each morning at 20 minute intervals over a period of 2 hours for 2 days. Then they were given intramuscular injections of sterile saline and the blood pressure readings were taken as before. This procedure was continued for 4 days. When no systolic reading above 100 and no diastolic reading above 65 was obtained, the patients were considered

<sup>1</sup> Ferrebee, J. W., Ragan, C., Atchley, D. W., and Loeb, R. F., *J.A.M.A.*, 1939, **113**, 1725.

<sup>2</sup> Grollman, Arthur, *Essentials of Endocrinology*, Philadelphia, J. B. Lippincott Company, 1941, p. 317.

<sup>3</sup> Soffer, Louis J., *Diseases of the Adrenals*, Philadelphia, Lea and Febiger, 1948, p. 162.

<sup>4</sup> Hartman, Frank A., and Brownell, Katharine A., *The Adrenal Gland*, Philadelphia, Lea and Febiger, 1949, p. 223.

\* This study was aided by grants from the Martha Leland Sherwin Fund and the Upjohn Company.

The desoxycorticosterone acetate used in this study was supplied by Dr. Kenneth W. Thompson of Organon, Inc.

† Clayton Foundation Fellow.

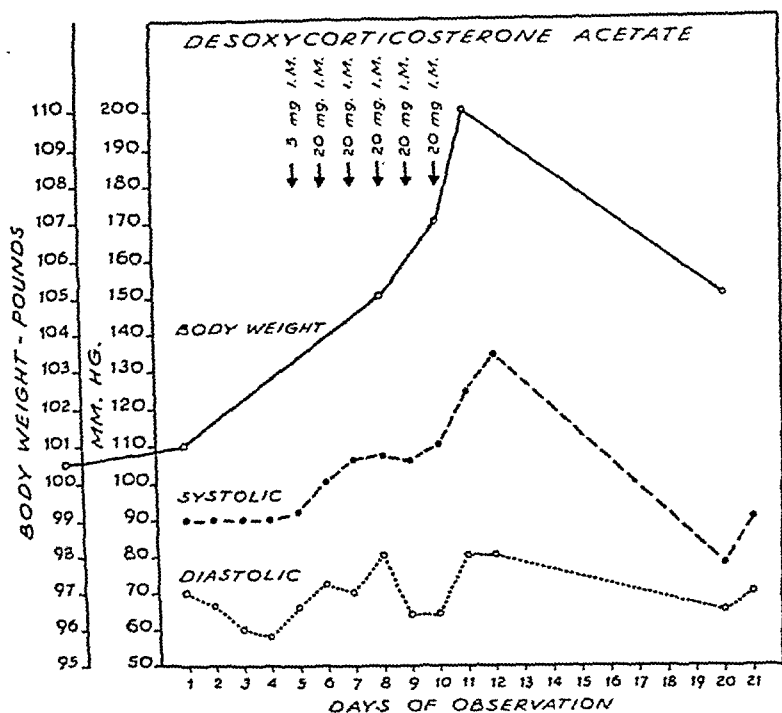


FIG. 1.

Shows the effect of the injection of 105 mg of desoxycorticosterone acetate over a period of 6 days. The diastolic and systolic blood pressure and the body weight increased. Note the drop in the blood pressure determinations and the body weight following cessation of administration of the desoxycorticosterone acetate. The increase in body weight was associated with swelling of the ankles which decreased as soon as the material was discontinued.

to be in a suitable condition to initiate injections of desoxycorticosterone acetate. It was injected intramuscularly early in the morning and a series of blood pressures was taken as before. The first day each of the 3 patients was given 5 mg of desoxycorticosterone acetate intramuscularly; the second day 1 of the patients was given 5 mg and 2 were given 20 mg each; the third day 1 was given 10 mg and 2 were given 20 mg each. On the fourth, fifth, and sixth days the dosage in each case was as shown in Fig. 1. On the third day following the injection of the initial dose of desoxycorticosterone acetate, the patients began to complain of tightness of the skin over the ankles. Fig. 1, which is representative of the 3 cases, illustrates the effect of desoxycorticosterone acetate on the systolic and diastolic pressures and on the body weight.

**Summary and conclusions.** Following the administration of desoxycorticosterone acetate

to persons with nutritive failure and hypotension, there was a prompt increase in blood pressure readings and in body weight (the increase in body weight is associated with retention of sodium in the tissues and this problem is being studied further). When the administration of this substance was discontinued, the blood pressure readings promptly returned to normal. During the first week after the administration of desoxycorticosterone acetate was begun, one patient stated that she had great symptomatic improvement; one complained of severe headaches, and she developed a "moon-faced" appearance which gradually disappeared when the injections were discontinued; the third patient noticed only swelling of the ankles. While our findings are of general interest, we do not recommend the use of desoxycorticosterone acetate as a therapeutic agent for hypertension.

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# Effect of Adrenal Cortex Extract on Blood Cells of the Embryonic Chick. (17435)

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In a previous paper,<sup>1</sup> it was shown that a transient lymphopenia and concomitant leukocytosis occurs in the adult fowl after a single injection of adrenal cortical extract, thus resembling mammalian blood cell changes reported by others.<sup>2</sup>

The present study was undertaken to determine the response of embryonic blood cells to adrenal cortex extract (ACE). In addition, other effects of the extract were observed in the hope that we might add to the meagre information known on the role of the adrenal cortex in the embryo.<sup>3</sup>

**Method.** Single Comb White Leghorn chick embryos were used. Sterile 0.85% saline (control) and aqueous adrenal cortex extract\* were injected into the allantoic cavity of embryos incubated 13, 14, 15 days, the dosage varying from 0.2 to 0.6 cc. Each embryo received only a single injection. One embryo series was bled from the vitelline vein<sup>4</sup> 24 hours after injection, while the second series was injected but allowed to hatch. In the first group, each embryo was bled once, and after bleeding was weighed to the nearest tenth gram, with extra-embryonic material removed.

Blood was diluted to the 101 mark in a certified RBC diluting pipette, the diluent<sup>5</sup> consisting of 25 mg of neutral red solution added to 100 cc of 0.85% saline. Total absolute counts per cubic millimeter (mm<sup>3</sup>) of erythrocytes and leukocytes combined, were made in a Neubauer Brightline Counting

Chamber.

Blood smears were stained with Wright's solution and 200 fields were examined on each smear. A Whipple disc was employed to determine the average number of cells in a field. Using a simple proportion, the actual count of a particular cell on a smear was converted to an absolute count:

$$\frac{\text{Smear count}}{\text{Avg No. cells in field} \times 200} = \frac{\times (\text{Absol. No. cells/mm}^3)}{\text{Total absol. count/mm}^3}$$

In the second series, the hatched chicks were bled on alternate days from the ulnar vein, over a period of one month after hatching, and the weight of every specimen taken after each bleeding. Smears were stained in the usual manner and differential white blood cell counts were made by counting 200 leukocytes in equally distributed sections of each slide.

**Results.** The injection of 0.3 cc of ACE had no apparent effect on the number of lymphoid cells in the peripheral blood of chick embryos of 14 to 16 days incubation. However, the hormone had a potent influence on the polymorph leukocyte number (Table I). Applying the chi-square test<sup>6</sup> to the frequency distribution of lymphoid cells (lymphocytes and hemocytoblasts<sup>7</sup>) and polymorph leukocytes, showed that chance accounted for the lymphocyte distribution, whereas the presence of the cortical extract significantly affected the distribution of the leukocytes.

The blood cell count means are shown in Table II. A statistical analysis of the difference between the means of the saline and ACE groups was not undertaken because of

<sup>1</sup> Shapiro, A. B., and Schechtman, A. M., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 440.

<sup>2</sup> White, A., and Dougherty, T. F., *Ann. N. Y. Acad. Sci.*, 1946, **46**, 859.

<sup>3</sup> Landauer, W., *Endocrinology*, 1947, **41**, 489.

\* The extract was furnished through the courtesy of Dr. David Klein of the Wilson Laboratories.

<sup>4</sup> Zifferblatt, A. N., and Seelaus, H. K., *Anat. Rec.*, 1931, **48**, 367.

<sup>5</sup> Forkner, C. E., *J. Exp. Med.*, 1929, **50**, 121.

<sup>6</sup> Waugh, A. E., *Elements of Statistical Method*, McGraw-Hill Book Co., 1943.

<sup>7</sup> Fennell, R. A., *J. Agric. Res.*, 1947, **74**, 217.

TABLE I.

Effect of 0.3 cc ACE on Lymphocytes and Polymorph Leukocytes of Embryonic Chicks Injected on the 13th, 14th, and 15th Day of Incubation and Bled 24 Hours Later. Application of the Chi-Square Test.

Class mark (No. cells/mm <sup>3</sup> )	Observed frequency (f)		Expected frequency (f')		(x <sup>2</sup> ) (f-f') <sup>2</sup> /f'	
	Sal.	ACE	Sal.	ACE	Sal.	ACE
Lymphocytes						
0 to 251	24	30	25.1	28.9	.048	.042
252 to 751	23	26	22.8	26.2	.002	.002
752 to 1251	9	10	8.9	10.1	.001	.001
1252 to 2251	12	10	10.2	11.8	.320	.271
2252 to 9751	7	10	7.9	9.1	.102	.089
Totals	75	86	74.9	86.1		.878*
Polymorphonuclear leukocytes						
0 to 101	48	39	40.4	46.6	1.420	1.230
102 to 301	9	10	8.8	10.2	.004	.004
302 to 2101	8	26	15.8	18.2	3.850	3.340
Totals	65	75	65.0	75.0		9.848†

\*  $\chi^2 = .878$ , 4 degrees freedom:  $P = .95$  or chance accounted for 95% of the observed frequency distribution.

†  $\chi^2 = 9.848$ , 2 degrees freedom:  $P = .01$  or ACE accounted for 99% of the observed frequency distribution.

TABLE II.

Effect of Single Injection of ACE and Saline (0.3 cc each) on the Blood Count of Embryos, Bled on the 14th, 15th, and 16th Day of Incubation (Injected 24 Hours Previously). Mean Values.

Cell (No./mm <sup>3</sup> )	14 days		15 days		16 days	
	Sal.	ACE	Sal.	ACE	Sal.	ACE
Lymphocyte	1,978	1,791	1,197	924	619	729
S.D.*	2,800	1,902	708	694	606	647
No. embryo	(27)	(34)	(16)	(18)	(32)	(34)
Polymorphs	106	309	108	319	152	416
S.D.	138	421	282	302	174	484
No. embryo	(17)	(23)	(16)	(18)	(32)	(34)
RBC† × 10 <sup>3</sup>	1,926	1,816	1,844	1,889	1,961	1,845
S.D.	268	297	187	229	334	328
No. embryo	(30)	(36)	(16)	(18)	(32)	(35)

\* S.D. =  $\frac{1}{N} \sqrt{N \sum (x^2) - \sum (x)^2}$ , where  $N$  is the number of embryos, and  $x$  is the individual value of the mean.

† is equivalent to number of total cells in counting chamber.

the large standard deviation (S.D.) and the skewness of the distribution of the individual counts. The erythrocytes do not seem to have been affected by ACE since the mean total absolute count, consisting mainly of erythrocytes, was approximately the same in both treated groups during the incubation period considered.

Table III shows that the hormone did not

affect embryo weight significantly, although a slight trend toward a weight loss was present in the 14 day hormone-injected embryos.

The presence of ACE depressed the hatchability rate of embryos in the second series (injected and allowed to hatch). Out of 154 saline-treated embryos, 82.4% hatched, while 68.4% of 149 hormone-injected embryos hatched. The critical ratio of the per cent



TABLE III.

Effect of Single Injections of ACE and Saline (0.3 cc each) on the Wet Weight of Embryos Weighed 24 Hr After Injection on the 14th, 15th, and 16th Day of Incubation. Mean Values.

	14 days		15 days		16 days	
	Sal.	ACE	Sal.	ACE	Sal.	ACE
No. embryos	(47)	(60)	(16)	(18)	(38)	(38)
Wet weight (g)	8.1	6.3	12.3	11.1	13.1	13.1
S.D.	1.0	1.2	0.9	1.0	1.1	1.5
S.E.*		1.53		1.33		1.85
t		1.22		.90		.56

\* S.E. (standard error) =  $\sqrt{\sigma M_1^2 - \sigma M_2^2}$ , where  $\sigma$  is the standard deviation of  $M_1$  and  $M_2$ , the means.  $t = \frac{M_1 - M_2}{S.E.}$ ,  $t = 2.2$  is significant.

difference was 2.86 which is statistically significant, since a ratio of 2.57 is equivalent to 99% confidence.

No discernible differences in the blood smears or weights of the hatched chicks between the 2 treated groups were observed. An apparent greater degree of barren skin area under the wings and abdomen and whiter down feathers were noticed in the ACE hatched chicks as compared to the control group.

*Discussion.* In the present study no change could be detected in the number of lymphoid cells after administration of adrenal cortical extract. However, there was a significant leukocytosis upon hormonal injection.

Failure to detect a transient lymphopenia is not in agreement with previous work on the adult fowl and mammals,<sup>1,2</sup> but we do not believe that such a conclusion is warranted in the present study. The relatively small number of lymphocytes on a smear and the resemblance of their precursors (hemocytoblast) to certain primitive erythrocytic cells,<sup>7</sup> renders the lymphocyte count less reliable than the counts of the more readily identifiable polymorphs. Furthermore, the large individual variation in lymphocyte numbers in the embryos would tend to obscure the hormonal effect.

Our failure to observe a lymphopenia after ACE injection might also be attributed to the length of time (24 hours) elapsing between injection of the hormone and removal of blood samples. Lymphopenia in adult mammals and fowl occurs at about 3-6 hours post injection.

No lymphopenia was observed in 15 embryos from which blood was removed at intervals of 3, 6, or 9 hours after injection of ACE. The period of 24 hours was selected for most of the specimens in order to allow for a greater degree of absorption from the chorioallantois, although the possibility remains that a shorter period might have been more effective.

Erythrocytes showed no response to ACE which corresponds with results in the adult fowl.<sup>1</sup> Variation of the dose from 0.2 to 0.6 cc had no apparent effect on the general embryonic blood picture.

The weight loss of embryos injected with adrenal cortical extract, although not statistically significant, taken together with the definite decrease in hatchability, suggests that the hormone retarded embryonic metabolism. This may have been due to the extract's toxicity or perhaps to its protein catabolic action.<sup>8</sup> Landauer,<sup>3</sup> reported that the injection of either 0.02 or 0.05 cc of ACE into 0 or 120 hour chick embryos produced a very marked retardation of growth and, with the larger amount of hormone, none of the embryos completed development. The use of 11-desoxycorticosterone or other steroids having no effect on protein metabolism,<sup>8</sup> may reveal whether ACE is toxic, as it seems to be, to the embryonic organism.

Thiourea injected into chick embryos has been reported<sup>9</sup> to inhibit the thyroid, lower

<sup>8</sup> White, A., *Proteins and Amino Acids in Nutrition*, Reinhold Publ., 1948, ch: 7.

<sup>9</sup> McMenamin, J. W., unpublished M.A. Thesis, Univ. Calif., Los Angeles, 1946.

body weight and produce a feather syndrome similar to the one here reported. This suggests that ACE may act indirectly on the embryo by depressing the thyroid to the extent of interfering with local feather and general embryonic metabolism.

**Summary.** 1. The blood picture of 14, 15 and 16 day White Leghorn chick embryos injected into the allantois with aqueous adrenal cortex extract, shows no significant change in the number of lymphocytes but a marked absolute polymorph leukocytosis, 24 hours after injection. The erythrocytes are not affected by the extract.

2. Large individual variation in the lymphocyte count and greater difficulty involved in their identification may have obscured the effect of the hormonal preparation on the lymphocyte count.

3. ACE treatment induced a tendency (not statistically significant) toward weight loss in the 14 day embryos, a significant decrease in the hatchability rate, and an apparent defective feather development. However the blood picture and weight in comparison with the controls was normal after hatching.

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## Glycerophosphatases of the Normal and Tumorous Frog Kidney. (17436)

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Lucké<sup>1</sup> described a neoplastic growth in the kidneys of the leopard frog, *Rana pipiens* Schreber (Fig. 1), which was manifested sometimes as a circumscribed adenoma and, at other times, as an adenocarcinoma with considerable infiltrative and destructive activities. The incidence, as reported by Lucké<sup>1</sup> and corroborated by the present studies, may be as much as 2% among both sexes.

It has been demonstrated that the alkaline phosphatase of the kidneys of many animals shows considerable activity.<sup>2,3</sup> Since it is further known that the phosphatase content of an organ is frequently altered under various pathological conditions<sup>4,5</sup> and especially in neoplasia<sup>6</sup> it was felt that studies of the alkaline and acid phosphatase systems might add to our knowledge of the physiology of the normal and pathological frog kidney.

**Experimental procedure.** Kidneys, both normal and tumorous were treated with a modified Gomori method recommended by Kabat and Furth.<sup>7</sup> Pieces of fresh kidney were fixed in cold acetone at 0°C for 24 hours and embedded in paraffin-Bayberry wax. Sections cut at 5  $\mu$  were mounted, deparaffinized and transferred to the buffered sodium glycerophosphate mixture and incubated for 8 hours at 37°C. Control sections were maintained. Some sections were stained in the usual way with Harris' hematoxylin and eosin; others were stained with safranin.

Tissues which were to be compared, namely, normal, neoplastic and calcium controlled sections were incubated and stained simultaneously.

Acid phosphatase was demonstrated by the Wolf, Kabat, and Newman<sup>8</sup> modification of Gomori's original method.<sup>9</sup>

Stewart<sup>10</sup> has pointed out that in cross sec-

<sup>1</sup> Lucké, B., *Am. J. Cancer*, 1934, **20**, 352.

<sup>2</sup> Kay, H. D., *Biochem. J.*, 1928, **22**, 855.

<sup>3</sup> ———, *Physiol. Rev.*, 1932, **12**, 384.

<sup>4</sup> Brain, R. T., and Kay, H. D., *Biochem. J.*, 1927, **21**, 1104.

<sup>5</sup> Folley, S. J., and Kay, H. D., *Ergebn. d. Enzymforschung*, 1936, **5**, 159.

<sup>6</sup> Greenstein, J. P., *J. Nat. Cancer Inst.*, 1943, **4**, 275.

<sup>7</sup> Kabat, E. A., and Furth, J., *Am. J. Path.*, 1941, **17**, 303.

<sup>8</sup> Wolf, A. E., Kabat, E., and Newman, W., *Am. J. Path.*, 1943, **19**, 423.

<sup>9</sup> Gomori, G., *Arch. Path.*, 1941, **32**, 189.

<sup>10</sup> Stewart, S. G., *Anat. Rec.*, 1927, **30**, 259.



FIG. 1

FIG. 2

FIG. 3

FIG. 1. Bilateral tumor (T34) of a male *Rana pipiens* fixed in Kaiserling fluid.  $\times 10$ .

FIG. 2. Alkaline phosphatase activity in the normal frog kidney (N6). The proximal convoluted tubules (at the right) show a +++ reaction, the glomeruli a +++ reaction, and the distal tubules (at the left) are negative. Safranin stained.  $\times 110$ .

FIG. 3. Alkaline phosphatase activity in the tumorous frog kidney (T25). The gland-like tubules of the tumorous (upper) portion show a negative phosphatase reaction, whereas the tubules of the adjacent non-tumorous portion (lower quarter) indicate a reduced enzyme activity from that seen in the normal (Fig. 2). Safranin stained.  $\times 110$ .

tion the frog's kidney is approximately hemicircular and may conveniently be divided into 3 crescent-shaped segments—the dorsal, intermediate and ventral zones. The proximal convolutions are quite uniformly restricted to the dorsal zone in which is located practically the entire system of collecting tubules. The distal convolutions were mainly found to lie in the ventral zone. In the central zone are grouped the glomeruli along with the transitions of the proximal to distal convolutions. Our results were tabulated using this description of the locations of the various kidney segments.

The intensity of the phosphatase reaction was graded by a system of pulses based on the size, distribution and density of granules in several sections: ++++ maximum reaction, *i.e.*, one in which the phosphatase deposition was so dense that the nuclear outline was just discernible, +++ large amount, ++ moderate, + slight, - negative.

**Results. 1. Sites of alkaline phosphatase activity in normal and tumorous kidney.** Alkaline phosphatase was consistently found to be heavily concentrated at the luminal borders of the proximal tubules and in the collecting ducts. A fair amount of the enzyme was also found in the glomeruli. However, the

glomerular capsule and the distal convolutions were always found to be negative (Fig. 2).

There was no difference in the alkaline phosphatase picture between the male and female kidneys, neither did there appear any preferential staining for any portion of the same kidney, *i.e.*, serial sections of normal kidneys excluded the possibility of local variations in phosphatase activity.

The alkaline phosphatase content of tumor-bearing kidneys is quite unlike that of their normal controls. In the first place the neoplastic portions of all affected kidneys showed a negative alkaline phosphatase reaction. Secondly, areas adjacent to the neoplastic growth, although exhibiting the same general distribution of alkaline phosphatase as the normal controls, showed a marked reduction in enzymatic activity (Table I and Fig. 3).

It is noted in Table I, that the nontumorous portions of some tumor-bearing kidneys (36 and 39) showed a slightly greater phosphatase reaction than did the others. Upon examination we found that these tumors were simply solid masses of cells without lumina. This would indicate that the frog renal carcinoma does not exert its influence on the surrounding kidney tissue phosphatase in its earlier stages, *i.e.*, before the tumor has dif-

TABLE I.  
Kidney Alkaline Phosphatase.

Frog expt.	Tumor	Non-tumorous portions of kidney				
		Proximal tubules	Collecting tubules	Glomerulus	Glomerular capsule	Distal tubules
1-14		++++	Normal ++++	+++	—	—
1-35 37-38, 40	—	+ to +++	Tumorous + to +++	+ to +++	—	—
36 and 39 Controls (technical)	—	+++	+++	++	—	—

The intensity of the phosphatase reaction was graded as follows: ++++ maximum, +++ large amount, ++ moderate, + slight, — negative.

ferentiated into the tubular form.

In all cases kidney sections at a distance from the neoplastic area showed a normal phosphatase content.

2. *Sites of acid phosphatase activity in normal and tumorous kidney.* Even when activated by 0.01 M manganous sulfate,<sup>11</sup> all portions of both the normal and neoplastic kidneys gave negative acid phosphatase reactions, except for an occasional positive glomerular reaction due to the phosphatase of the red blood corpuscles. These findings are comparable to those of other investigators<sup>8,9</sup> for normal kidneys.

*Discussion.* In all cases reported<sup>7,12-14</sup> the proximal tubules always show a strong positive reaction, whereas the distal convolutions are generally negative. The glomeruli are usually entirely negative. Only the glomeruli of the cat kidney<sup>12</sup> shows a positive alkaline phosphatase reaction similar to that which we observed in the frog glomeruli.

The high concentration of alkaline phosphatase in the proximal tubules suggests the possible role of this enzyme in sugar reabsorption since it has been shown that these tubules are the site of glucose reabsorption in the normal kidney.<sup>15</sup> Then too, it is well known

that phlorhizin inhibits alkaline glycerophosphatase and also prevents the active reabsorption of glucose in the amphibian kidney.<sup>16</sup> A scheme for such a glucose-reabsorbing mechanism has been presented for certain mammalian kidneys.<sup>14</sup>

Our observation of a complete lack of alkaline phosphatase in the tumorous portions of the affected frog kidney, and its marked depletion in the adjacent renal tissue, indicates that the neoplasm interferes in some way with the metabolism of the surrounding tissue even though there is no microscopic evidence of its infiltration.

It must be emphasized that our results are based solely on the use of sodium-beta-glycerophosphate as the phosphoric ester hydrolyzed by the phosphatase of the kidney. The work of Dempsey and Deane<sup>17</sup> indicates that certain regions of the duodenum contain substrate-specific phosphatases, which suggests the possibility that other phosphatases might very well be demonstrable in the neoplastic amphibian kidney if other substrates and different pH ranges were employed.

*Summary.* It has been demonstrated for the first time, histochemically, that the normal kidney of the frog, *Rana pipiens*, possesses a strong alkaline glycerophosphatase reaction

<sup>11</sup> Moog, F., *J. Cell. and Comp. Physiol.*, 1943, **32**, 95.

<sup>12</sup> Gomori, G., *J. Cell. and Comp. Physiol.*, 1941, **17**, 71.

<sup>13</sup> Bourne, G., *Quart. J. Exp. Physiol.*, 1943, **32**, 1.

<sup>14</sup> Wilmer, H. A., *Arch. Path.*, 1944, **37**, 227.

<sup>15</sup> White, H. L., and Schmitt, F. O., *Am. J. Physiol.*, 1926, **76**, 220.

<sup>16</sup> Walker, A. M., and Hudson, C. L., *Am. J. Physiol.*, 1937, **118**, 130.

<sup>17</sup> Dempsey, E. W., and Deane, H. W., *J. Cell. and Comp. Physiol.*, 1946, **27**, 159.

in its proximal tubules, especially at their brush borders, whereas the distal convolutions are negative. The glomeruli show a moderate alkaline reaction, categorizing the frog in a class with the cat (the only other animal thus far investigated that gives this reaction). With regards to the neoplastic kidney, the tumor cells themselves are alkaline

phosphatase negative with an accompanying depletion of the enzyme in adjacent non-tumorous portions of the renal tissue.

Both normal and tumorous kidneys are essentially negative for acid glycerophosphatase. The significance of these results is discussed.

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## Depressing Effect of Inositol on Serum Cholesterol and Lipid Phosphorus in Diabetics.\* (17437)

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The problem of the relation of serum cholesterol levels to human atherosclerosis is much debated. An agent capable of depressing serum cholesterol levels in hypercholesteremic individuals would offer a new approach to its study. There is some evidence to show that inositol is such an agent.

McHenry and Patterson<sup>1</sup> suggested that inositol has a specific effect on cholesterol metabolism; they pointed out that it is an effective lipotropic agent particularly when large amounts of cholesterol are present in the liver. Herrmann<sup>2</sup> found that inositol depressed blood levels of cholesterol and chole-

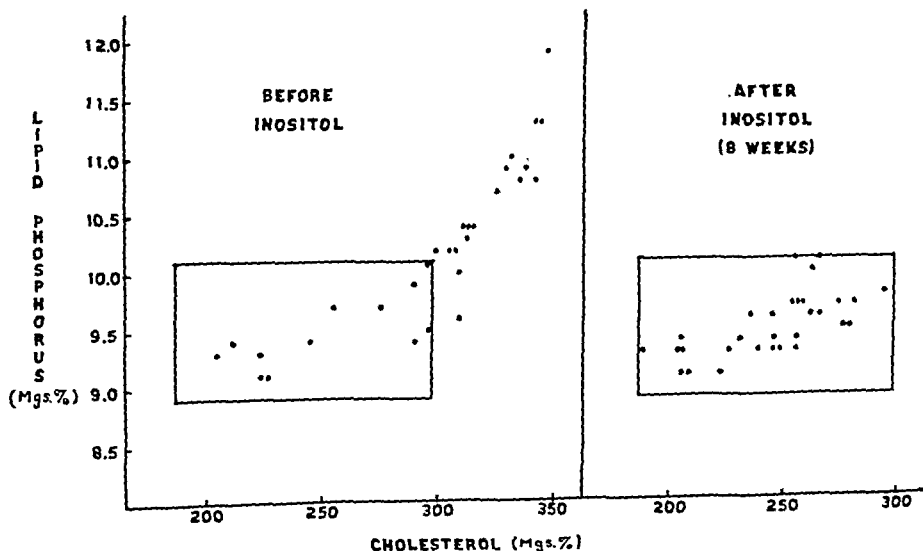


FIG. 1.

Values of serum total cholesterol and lipid phosphorus for 30 diabetic patients before and 8 weeks after inositol. Enclosed areas represent range of normal.

\* This study was supported in part by a grant from Commercial Solvents Corporation.

<sup>1</sup> McHenry, E. W., and Patterson, J. M.,

*Physiol. Rev.*, 1944, 24, 129.

<sup>2</sup> Herrmann, G. R., *Proc. Soc. Exp. Biol. and Med.*, 1946, 63, 436.

terol esters in old hens. Gephardt<sup>3</sup> found that in one case of xanthomatous biliary cirrhosis doses of 3 g of inositol per day for 3 months produced a marked drop in total lipids and some drop in cholesterol and cholesterol esters; the patient, however, received a low-fat diet concurrently. On the other hand, Shay<sup>4</sup> states that doses of 1.2 g a day for 3 weeks had no effect on blood cholesterol in human diabetics. Herrmann<sup>5</sup> found that 20 hypercholesteremic patients given 2 g of inositol a day for 25-30 days had an average drop of 19% and 17% of cholesterol and cholesterol esters respectively; one showed no change: he found also that there was a tendency for phospholipids to rise.

The action of inositol on serum lipids and on the atherosclerotic process is being studied in this hospital. The present paper reports its effect on certain serum lipids in a group of diabetic patients.<sup>†</sup>

**Procedure.** Total cholesterol and cholesterol esters<sup>6</sup> and lipid phosphorus<sup>7</sup> were determined on the blood serum of all experimental subjects. Similar determinations were made on 100 control subjects, consisting of staff physicians, nurses and hospital patients not known to have any disease affecting cholesterol metabolism.

Of the 30 diabetic patients used, 13 were selected because of high blood cholesterol levels previously established in this clinic; the remainder were picked at random from the diabetic clinic provided they fulfilled the criteria of fixed diet, fixed insulin dosage and adequate diabetic control for 6 months prior to beginning the experiment. Repeated de-

TABLE I. The Mean, Range, Standard Deviation, and Standard Error of the Mean of Total Cholesterol, Cholesterol Esters and Lipid Phosphorus in Normals and in Diabetics Before and After Taking Inositol											
	Total cholesterol, mg %				Cholesterol esters, mg %				Lipid phosphorus, mg %		
	Mean	S.D.	S.E.	Range	Mean	S.D.	S.E.	Range	Mean	S.D.	S.E.
Normals (100)	236.3	±24.0	±2.4	(188-297)	167	±16.8	±1.7	(137-204)	9.29	±0.20	±0.02
Diabetics (30)											
Control	294	±43.5	±8.0	(205-346)	208	±29.6	±5.4	(144-246)	10.2	±0.71	±0.13
Inositol: 1 week	268	±33.9	±6.2	(198-321)	189	±24.0	±4.4	(138-225)	10.0	±0.61	±0.11
" "	254	±31.3	±5.7	(188-303)	178	±22.6	±4.1	(136-213)	9.7	±0.42	±0.08
" 4 "	247	±27.7	±5.1	(186-292)	173	±19.3	±3.5	(129-207)	9.5	±0.31	±0.06
" 8 "	244	±26.8	±4.9	(189-293)	171	±18.7	±3.4	(131-208)	9.5	±0.27	±0.05

<sup>3</sup> Gephardt, M. C., *Ann. Int. Med.*, 1947, **26**, 764.

<sup>4</sup> Shay, H., in discussion of Vorhaus, M. G., Gompertz, M. L., and Feder, A., *Am. J. Dig. Dis.*, 1943, **10**, 48.

<sup>5</sup> Herrmann, G. R., *Exp. Med. and Surg.*, 1947, **5**, 149.

<sup>†</sup> The authors are indebted to Dr. John H. Keating for his helpful advice and to Miss Stephanie Ilka for technical assistance in this investigation.

<sup>6</sup> Sperry, W., and Brand, F. C., *J. Biol. Chem.*, 1943, **150**, 315.

<sup>7</sup> Youngburg, G. E., and Youngburg, M. V., *J. Lab. and Clin. Med.*, 1930, **16**, 158.

terminations on these 2 diabetic groups were practically identical as far as mean, range and standard deviation of cholesterol fractions and lipid phosphorus were concerned; they are therefore considered as one unit.

At least 2 baseline total cholesterol, cholesterol esters and lipid phosphorus determinations were made on all diabetic patients before they were given inositol. They then began taking 3 g of inositol daily (two 0.5 g capsules after each meal); bloods were taken at intervals of 1, 2, 4, and 8 weeks thereafter.

**Results.** The range of normals is indicated by the enclosed area in Fig. 1. The mean, range and standard deviations are shown in Table I. No apparent variation was found with regard to age or sex and repeated determinations on the same subject showed little or no variation over a period of several months.

Before taking the inositol, the diabetic patients tended to show elevated serum total cholesterol, 18 (60%) being outside the range of normals as shown in Fig. 1, only 4 below the normal average, the rest high normal. Cholesterol esters were proportional to total cholesterol, the ratio of esters to total being relatively constant (68-73%). Lipid phosphorus tended to be high when the total cholesterol was high, but the correlation was not as consistent as with the cholesterol esters. No correlation between the level of cholesterol fractions or lipid phosphorus and the severity of diabetes or the accuracy of its control could be made.

In all of the diabetic patients fed the inositol a fall occurred in the serum cholesterol and lipid phosphorus. After 8 weeks (Fig. 1) all determinations were within the normal range. As shown in Table I, the total cholesterol tended to fall abruptly over the first 2 weeks, then to fall more slowly or become stationary. The higher initial total cholesterol showed the greater tendency to fall; thus, 17 diabetics with an initial total cholesterol above 300 mg% had an average drop of 69.2 mg% in 8 weeks, while the 13 diabetics below 300 mg% had an average drop of only 29.0 mg%. The proportion of esters to the total remained constant after inositol, and lipid phosphorus tended to fall in conjunction with fall in total cholesterol, contrary to Herrmann's findings. When the patients were taken off inositol, a gradual rise occurred in the determined serum lipids, but after 6 weeks none had returned to the baseline levels.

The only possible untoward effect of inositol observed occurred in one woman who complained of gastric distress; many of the patients reported a feeling of increased vigor and well-being. No consistent change in the diabetic status of any of the patients was noted.

**Conclusions.** Inositol is an effective agent in lowering serum cholesterol and lipid phosphorus in hypercholesteremic diabetics. Further study of its action and applications is warranted.

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## Metabolism of Some 9-Aminoacridine Derivatives. (17438)

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Many compounds in the 9-aminoacridine series have been synthesized for the purpose of studying their action against a variety of parasitic infections, and as disinfectants. The recent antimalarial survey<sup>1</sup> included a large number of these compounds. One of them was Acranil (SN-186), which was found to be only

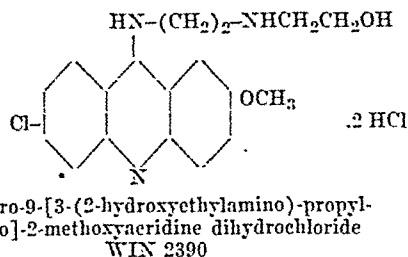
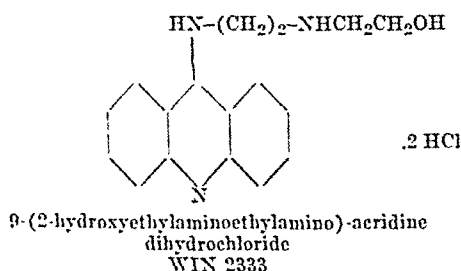
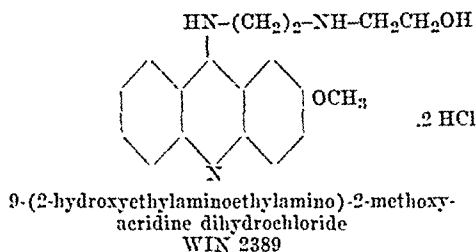
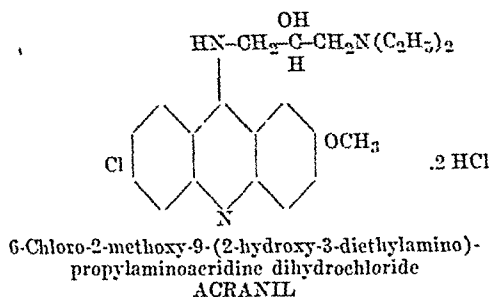
slightly active against avian malaria.<sup>2</sup> Previously<sup>3-6</sup> it had been used successfully for

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<sup>1</sup> Wiselogle, F. Y., *A Survey of Antimalarial Drugs*, J. W. Edwards, Ann Arbor, 1946, pp. 1321-1377.

<sup>2</sup> Gingrich, W. D., and Fillmore, R. S., *Am. J. Hyg.*, 1942, 36, 276.

the treatment of human giardia infections and, more recently, against *Hymenolepis nana*.<sup>7</sup> Berberian has also found it quite effective for the treatment of human subjects infested with *Enterobius vermicularis*. Three closely related compounds which are superior to Acranil against oxyurids of mice,<sup>8</sup> and also as to acute toxicity for mice\* are Win 2333, 2389, 2390. The structural formulae of these 4 compounds, to be discussed in this paper, are as follows:



Some information on the absorption, excretion, and tissue distribution of a typical 9-aminoacridine derivative is available as a result of the widespread interest in the use of Atabrine<sup>†</sup> for the therapy of human malaria.<sup>9-12</sup> It is known, for example, that the highest tissue concentrations for Atabrine, following oral administration to rats, are found in the liver, with lesser amounts in the spleen, lung, and kidney, in order of decreasing concentration.<sup>9,10</sup> In dogs, deposition in the tissues accounts for 10 to 30% of the oral dose.<sup>10</sup>

We have studied the 4 compounds listed above with respect to their absorption, excretion, and tissue-distribution, since these factors are important in determining their suitability for the treatment of susceptible parasitic infections.

**Methods.** The experimental subjects were male hooded rats from our colony, weighing 250-365 g each. They were kept in metabolism cages, one pair to each cage. In order to simulate as closely as possible the conditions used for the treatment of intestinal parasitic infections, they were allowed free access to our regular colony diet and water until the medication was given, but then only water was provided for the remainder of the experimental period. The drugs were given by

<sup>†</sup> Registered trade-marked name of Winthrop-Stearns, Inc., brand of quinaerine.

<sup>9</sup> Barlow, O. W., Auerbach, M. E., and Rivenburgh, H., *J. Lab. Clin. Med.*, 1945, **30**, 20.

<sup>10</sup> Army Malaria Research Unit, *Ann Trop. Med. and Parasitol.*, 1946, **40**, 173, 181, 368, 372, 472, 482.

<sup>11</sup> Dearborn, E. H., Oldham, F. K., Geiling, E. M. K., and Kelsey, F. E., *J. Pharm. and Exp. Therap.*, 1943, **78**, 120.

<sup>12</sup> Annegers, J. H., Snapp, F. E., Paskind, L., Ivy, A. C., and Atkinson, A. J., *War Med.*, 1943, **4**, 176.

<sup>3</sup> deMuro, P., *Acta Med. Scand.*, 1939, **102**, 17.

<sup>4</sup> Grott, J. W., *Schweiz. Med. Wochschr.*, 1939, **69**, 683.

<sup>5</sup> Friederich, L. V., *Gastroenterol.*, 1940, **65**, 24.

<sup>6</sup> Berberian, D. A., *Am. J. Trop. Med.*, 1945, **25**, 441.

<sup>7</sup> Berberian, D. A., *Am. J. Trop. Med.*, 1946, **26**, 339.

<sup>8</sup> Berberian, D. A., and Dennis, E. W., manuscript in preparation.

\* Laboratory data, courtesy of Dr. J. O. Hoppe.



stomach tube in 5 cc of water per kg body weight. This was enough water to dissolve the drug except for some doses of Win 2389 and 2390, which remained partly in suspension.

Twenty-four hours after medication samples of blood were drawn into oxalate by heart puncture. The animals were then sacrificed; the carcasses were frozen and stored until they could be analyzed. Urine and feces were collected for the 24-hour experimental period, the samples thus representing the combined excretions of one pair of rats. The blood and tissue samples, however, were usually analyzed for each animal. The methods used for the extraction and analysis have been described by Brodie *et al.*,<sup>13,14</sup> with the exception that in this work ethylene dichloride was used as a solvent in place of petroleum ether. As a routine measure the extracts were washed with 10% NaOH to remove metabolic products of the drugs, but no colored material was removed in this way. Acranil, Win 2389, and Win 2390 fluoresce in the visible range; they were determined in the Coleman photofluorometer using the filter system B-2, PC-9A. Win 2333 fluoresces only in the ultraviolet range. However, its behavior proved to be too erratic to use this property for its determination, therefore a colorimetric method was used.<sup>†</sup> Since the sensitivity of the method was not as great as the fluorometric, it seemed inadvisable to attempt the determination of tissue concentrations of Win 2333 on any dose less than 200 mg/kg, and blood determinations were not made.

When the animals were dissected, the procedure was as follows: Aliquots of about 2 g of liver and lung were accurately weighed for extraction, also the whole spleen, and one

whole kidney. If the stomach contents were sufficiently solid, a 2 g aliquot was taken, otherwise the entire contents were washed with water into the vessel to be used for the extraction. The intestines were washed out in a similar way, and to these washings were added the cecal contents. The analytical results are presented in Table I.

**Discussion.** It is evident that the number of experimental animals used was not sufficient to establish the tissue concentrations of each drug at each dose level with great precision. In about 2/3 of the paired analyses the agreement was very close; in the other third the variations were greater than one might wish (*i.e.*, 100% or more). In about half the aberrant figures the extent to which absorption had proceeded was clearly a factor (for example, Acranil, 500 mg/kg pair). In some other cases the extent of absorption alone could not account for the differences between paired animals (for example, Win 2389, 500 mg/kg pair). However, the purpose of the work was only to establish general trends, and with this in mind, the following generalizations can be made:

1. The tissue concentrations tended to increase with the dose level, as would be expected.

2. The urinary and fecal excretions did not show any consistent relationship to the dose level. Doubtless this is a reflection of the fact that considerable, but variable, amounts of the drugs remained in the gastrointestinal tract. However, it can be said that Acranil was excreted to the least extent in the urine, and Win 2389 to the greatest. Acranil was also excreted in the feces to the least, and Win 2389 to the greatest extent.

3. The blood concentrations of Acranil and Win 2390 increased with the dose level, and were of the same magnitude for equivalent doses. The blood concentrations of Win 2389 averaged as high, but were in a mixed order.

4. At any dose level chosen for comparison, Acranil accumulated in the tissues more than any of the other compounds, and Win 2333 accumulated the least; the affinity of liver for Acranil was particularly marked. In order of decreasing over-all tissue affinity, and of decreasing concentration in the individual tis-

<sup>13</sup> Brodie, B. B., Udenfriend, S., and Baer, J. E., *J. Biol. Chem.*, 1947, **168**, 299.

<sup>14</sup> Brodie, B. B., Udenfriend, S., Dill, W., and Downing, G., *J. Biol. Chem.*, 1947, **168**, 311.

<sup>†</sup> The extraction, washing, and transference into 0.1 N HCl were carried out in the specified way except that 10 cc of the HCl was used instead of 6 cc. The color of the HCl solution was then read in an Evelyn colorimeter using a 420 filter, comparing with standards containing 0.005-0.5 mg of Win 2333 in 10 cc of 0.1 N HCl.

TABLE I.  
Absorption, Tissue Distribution, and Excretion of Some 9-Aminoacridines 24 Hr after Oral Administration to Rats.  
Amounts in tissues,<sup>†</sup> gastrointestinal tract, excreta, and blood.

Absorption, Tissue Distributions, and Metabolism in tissues, gastrointestinal tract, excreta, and blood.										
Compound, dose		Blood conc., mg/l	Stomach cont., mg	Intestinal and cecal cont., mg	Liver, mcg/g	Kidney, mcg/g	Spleen, mcg/g	Lung, mcg/g	% of dose in tissues and urine	% of dose in g. i. con- tents and feces
mg/kg	mg									
Acrinil										
50	13.0	1.1	0.07	0.52	430	75	100	105	36.0	7.4
50	13.5	0.6	1.03	0.02	450	98	130	42	34.0	11.0
50	27.6	1.3	4.35	3.57	485	163	400	150	20.0	29.0
100	27.6	1.3	0.52	1.46	172	166	300	330	8.6	6.5
500	124.0	3.8*	24.3	10.0	2000	368	1040	535	2.8	15.0
500	139.0		96.0	9.9	880	378	565	310	8.4	69.0
WIN 2333										
200	66	—	3.1	11.2	53	17	54	12	2.2	27
200	64	—	17.6	1.9	33	15	40	11	1.8	36
500	182	—	80.6	4.9	47	49	41	11	2.0	50
500	140	—	18.0	1.4	98	33	54	30	2.9	17
WIN 2380										
50	13.8	1.6	0.02	4.60	23	7	2	2	3.3	74
50	14.0	1.4	0.07	4.62	18	10	16	4	3.3	74
100	29.7	0.8	1.72	6.55	17	30	43	14	6.4	39
100	27.6	0.7	0.01	1.53	17	32	86	32	7.0	17
500	135.0	3.2*	73.2	5.2	147	86	364	117	2.3	63
500	138.0		57.0	5.9	61	56	71	58	1.4	51
WIN 2390										
50	14.2	0.2	0.01	2.20	47	47	45	14	4.2	24
50	14.6	0.2	0.001	3.64	25	55	71	8	2.7	33
50	14.0	0.3	0.09	4.20	72	37	65	16	6.5	37
50	15.0	0.4	3.10	1.70	238	67	125	17	18.0	38
100	30.2	0.8	5.36	6.42	143	145	90	26	7.3	52
100	29.5	1.0	7.16	5.80	123	100	390	35	5.6	53
100	27.4	0.4	0.01	1.75	147	38	37	16	6.9	22
100	27.4	0.4	0.00	6.24	148	42	37	20	3.7	38
200	52.0	0.5	20.1	10.30	227	139	143	163	7.2	63
200	52.8	1.0	18.1	10.30	568	174	148	100	10.0	58
500	124.0	3.8*	17.6	76.4	256	124	233	100	2.7	76
500	133.0		51.6	26.0	279	81	70	80	2.7	60

\* These animals were so close to a state of collapse that it was not possible to obtain sufficient blood for individual analyses.

† Tissue concentrations are based on wet weight.

sues the several compounds may be rated about as follows:

- a. Acranil; liver > spleen > kidney > lung
- b. Win 2390; liver > kidney > spleen > lung
- c. Win 2389; spleen > liver > kidney > lung
- d. Win 2333; liver > spleen > kidney > lung

It is of interest to compare the metabolism of these drugs with that of Atabrine, although the data available for comparison<sup>9</sup> were obtained with a higher dose than any used in this work: 675 mg/kg. None of these 4 drugs was absorbed as fast as Atabrine. Only at the lowest dose levels were as small (absolute) amounts found in the gastrointestinal tract as following Atabrine, and even less than 50 mg/kg of Win 2390 would have been required to produce an equivalent result. The reported tissue-distribution of Atabrine resembles that of Win 2389 in item<sup>4</sup> above. However, the latter compound was still only about half absorbed when the animals were killed. Win 2390 differed from Atabrine chiefly in giving higher liver concentrations. Even at the lowest dose level Acranil produced much higher liver concentrations than 675 mg/kg of Atabrine, and the other tissues contained amounts of the same order as following Atabrine.

It was noted that these compounds under-

go some chemical alterations, probably in the liver, followed by excretion through the bile. This was shown by the fact that the ethylene dichloride extracts of both the intestinal (plus cecal) contents and of the feces contained an intensely yellow non-fluorescent substance which remained in the organic solvent, even when the latter was extracted with N HCl. This indicates that the basic side chain had been lost, leaving a neutral compound, possibly an acridone. A similar metabolic process has been observed by Hawking and Frazer in their studies of Miracil D.<sup>15</sup>

*Summary.* The absorption, excretion, and tissue-distribution of 4 compounds of the 9-aminoacridine series have been studied in rats at oral dose levels ranging from 50 to 500 mg/kg. At any dose level Acranil concentrates in the tissues to the greatest extent, while the other compounds follow in this order: Win 2390, 2389, 2333. For any single compound, the tissue concentrations ordinarily increase with the dose, the largest amount being found in the liver. In order of increasing rate of absorption the compounds are Win 2390, Win 2389, Acranil, Win 2333 but none of these is as completely absorbed within 24 hours as has previously been reported for Atabrine.

<sup>15</sup> Hawking, F., and Ross, W. F., *Brit. J. Pharm. and Chemotherap.*, 1948, **3**, 167.

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## Regeneration of the Neural Portion of the Retina from Pigment Cells in Adult Urodele Eyes.\* (17439)

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In previous experiments on grafted adult salamander eyes, it has been shown by the author,<sup>1-3</sup> that degeneration of the neural elements of the retina is followed by regenera-

tion. The retina can be regenerated with sub-

<sup>1</sup> Stone, L. S., and Zaur, I. S., *J. Exp. Zool.*, 1940, **85**, 243.

<sup>2</sup> Stone, L. S., and Farthing, T. E., *J. Exp. Zool.*, 1942, **91**, 265.

<sup>3</sup> Stone, L. S., and Ellison, F. S., *J. E. Zool.*, 1945, **100**, 217.

\* Aided by grants from the James Hudson Brown Fund and the United States Public Health Service.

sequent return of vision at least 4 times in the same eye repeatedly transplanted.<sup>2</sup> The functional patterns associated with each of the retinal quadrants are reestablished and the visuomotor responses of the host are determined by the orientation of the graft.<sup>4-7</sup>

Regeneration of the retina in amphibians especially in larval urodeles has been reported previously by other investigators. As to its source of origin iris tissue has been claimed by some authors.<sup>8-10</sup> Retinal tissue at the ciliary margin<sup>8,11</sup> and ganglion cells in the fully differentiated retina<sup>12</sup> have been proposed as the source. One of the above mentioned investigators<sup>8</sup> believed that the regenerating retina in larval urodeles could also be derived from the pigmented epithelial layer.

An extensive study has been made to settle the question of the source of origin of the regenerating neural portion of the retina in the eye of the adult urodele, *Triturus v. viridescens*. In various groups of experiments over 500 grafts of iris tissue have been placed in the aqueous and vitreous chambers of the eye. None of them has given rise to retinal tissue. A detailed study was made of the degeneration and regeneration of the retina in 200 grafted eyes preserved at close intervals for at least 3 months after operation. Degeneration spreads throughout the neural retina during the first 3 weeks. In some cases destruction proceeds at different rates in various parts of the same eye and when degeneration overlaps early stages of regeneration it is more difficult to obtain a clear picture of the origin of the new retina. However, when degeneration is simultaneous in all parts it is clearly demonstrated that the new neural

portion of the retina derives its origin from the surviving retinal pigment cells. This fact was demonstrated also in a study of 100 eyes from which the entire neural retina was successfully removed intact through a dorsal slit along the corneo-scleral junction after it had been detached from the underlying retinal pigment layer by a gentle stream of Ringer's solution emerging from a micropipette. The excised retinæ were preserved and later served as controls for the eyes with regenerating retinæ.

As soon as the pigment epithelial cells lose contact with neural retinal tissue they can be followed step by step as they change their structure and function. At first they become flattened oval densely pigmented bodies. Soon they increase in size and undergo mitosis. One daughter cell migrates inward, loses its pigment and gives rise to a chain of cells which later form the new neural retina. The other takes on the characteristics of a retinal pigment cell. Its pigment granules regain their ability to migrate in the presence of light only when the rod and cone cells of the new retina above them differentiate. It was also noticed in these experiments that the lost vitreous body was eventually replaced.

In another group of 63 eyes various injuries were made by inserting a micropipette through a wound in the cornea and removing by suction limited areas of the neural portion of the retina. In the larger wound areas the denuded pigment cells responded in the same manner as in those eyes from which all of the neural retina was eliminated. On the other hand if the wound area was small a rapid proliferation and migration of the heavily pigmented cells partially filled the space before depigmentation spread among them. As in other wound areas repair was carried to completion to replace the lost retinal tissue. There was always a sharp demarcation between the surrounding intact neural retina and the regenerating one. No evidence was found to support the idea that surviving cells of the neural portion of the retina bordering the wound ever contributed to the formation of the new retina.

To bring forth a response from the retinal

<sup>4</sup> Stone, L. S., *Proc. Soc. Exp. Biol. and Med.*, 1944, **57**, 13.

<sup>5</sup> Stone, L. S., *Exp. Cell Research*, Sup. 1, 1949, 582, (*Proc. 6th Int. Cong. Exp. Cytol.*) 1947.

<sup>6</sup> Stone, L. S., *Ann. N. Y. Acad. Sci.*, 1948, **49**, 856.

<sup>7</sup> Sperry, R. W., *J. Comp. Neurol.*, 1943, **70**, 33.

<sup>8</sup> Waech, H., *Roux Arch.*, 1920, **46**, 328.

<sup>9</sup> Sato, T., *Roux Arch.*, 1933, **130**, 19.

<sup>10</sup> Monroy, A., *Roux Arch.*, 1939, **139**, 536.

<sup>11</sup> Zolokav, M., *Rev. Suisse d. Zool.*, 1944, **51**, 443.

<sup>12</sup> Bücklers, M., *Arch. f. Ophthalm.*, 1933, **130**, 257.

pigment cells it is not essential to destroy or completely remove the neural portion of the retina. If a segment of the fully differentiated or regenerating neural retina becomes detached as an elevated fold it may survive. In the arch beneath it the retinal pigment cells immediately give rise to a secondary neural retina.

In 59 experiments segments of the eye wall containing the retinal pigment layer were im-

planted in the vitreous chamber of recipient eyes from which the lens had been removed. They also showed the development of neural retina from the pigment layer. It is therefore concluded that the regenerating neural portion of the retina in the adult urodele eye has its only source of origin from the cells of the retinal pigment layer.

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### Enhancement of Hemolytic Activity of Complement by Polyethylene Glycols.\* (17440)

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In the course of an investigation designed to test the ability of various substances to prevent the deterioration of complement, it was found that certain of the polyethylene glycols greatly enhance the hemolytic activity of complement. The polyethylene glycols, a series of polymers with the general formula  $\text{HOCH}_2(\text{CH}_2\text{OCH}_2)_x\text{CH}_2\text{OH}$ , are soluble in water and in many of the aromatic hydrocarbons. The compounds with average molecular weight up to 700 are liquids; those with average molecular weight above 1000 are wax-like solids, sold under the trade name "Carbowax".<sup>†</sup> This paper constitutes a preliminary report on the effect of the polyethylene glycol carbowax-4000 on the hemolytic activity of complement, as determined by complement titrations in the presence and absence of this compound.

**Procedure.** Complement titrations were carried out according to the 50% hemolysis end-point method developed by Mayer *et al.*,<sup>1</sup> and Kent *et al.*<sup>2</sup> The reaction mixtures, 20

ml in volume, had the following composition: 1) 1 part complement, the dilution so adjusted as to yield final dilution values ranging from 1:3300 to 1:950; 2) 1 part standardized suspension of sensitized erythrocytes; 3) either 2 parts of buffered saline,<sup>3</sup> or 2 parts of an 8% solution of carbowax-4000 in buffered saline. The sensitized erythrocytes were well mixed with either buffered saline or carbowax-4000 solution, and then the complement added dropwise with constant agitation. All components were kept in ice-water until time of incubation. The reaction mixtures thus prepared were incubated at 37°C for 60 minutes, with frequent agitation. After centrifugation of the mixtures, readings of per cent hemolysis in the supernates were made at 5500 Å in a Coleman spectrophotometer, an optical density of .500 representing complete hemolysis. Titrations were carried out in duplicate. Controls were set up of 1) sensitized erythrocytes and carbowax-4000, 2) unsensitized erythrocytes and carbowax-4000, and 3) complement, unsensitized erythrocytes and carbowax-4000.

**Results and discussion.** None of the control tests showed any hemolysis. The results of the titrations are shown in Fig. 1, in which

\* This work was supported by a grant from the National Tuberculosis Association.

† Carbon and Carbide Chemicals Corporation, New York.

<sup>1</sup> Mayer, M. M., Eaton, B. B., and Heidelberger, M., *J. Immunol.*, 1946, **53**, 31.

<sup>2</sup> Kent, J. F., Bukantz, S. C., and Rein, C. R., *J. Immunol.*, 1946, **53**, 37.

<sup>3</sup> Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M., *J. Exp. Med.*, 1946, **84**, 535.

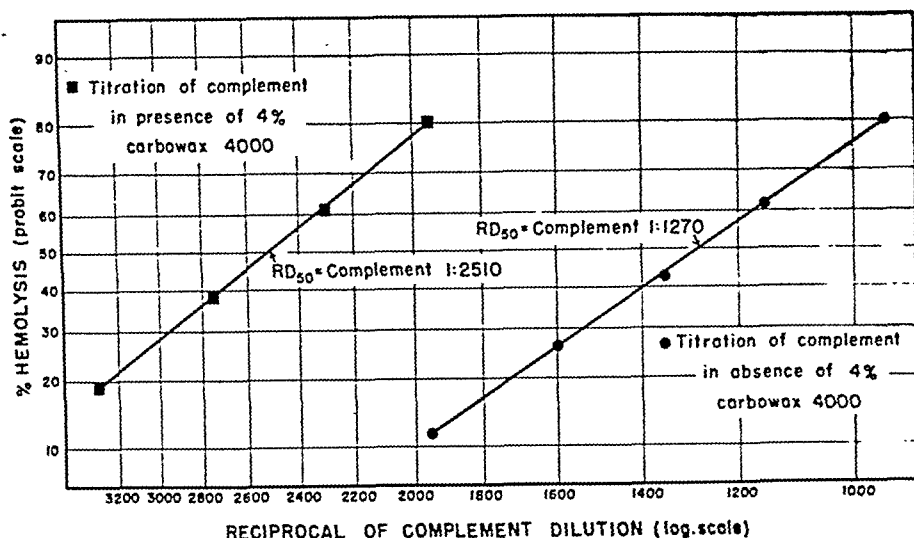


FIG. 1.

Titration of complement in the presence and absence of 4% carbowax-4000. The complement dilutions referred to on the abscissa represent final dilutions, not the dilutions added to the reaction mixture.

the probit of the percent hemolysis is expressed as a function of the log of the dilution of complement, in the presence and absence of 4% carbowax-4000. The dilutions of complement, with or without 4% carbowax-4000, calculated to yield a required percent hemolysis, as determined from the curves of Fig. 1, are set forth in Table I. A striking difference in the ability of a given dilution of complement to lyse sensitized erythrocytes in the presence or absence of carbowax-4000 is evident. Within the range of values for percent hemolysis which can be used with assurance in this technic, the two curves over-

lap only at a complement dilution of 1:1950. At this point, complement alone gives 11.5% hemolysis; complement and 4% carbowax-4000 together, give 79.9% hemolysis, an increase of nearly 700%. The  $RD_{50}$  unit (that amount of complement required to lyse 50% of the sensitized erythrocytes) in the absence and presence of 4% carbowax-4000 is 1:1270 and 1:2510 respectively. The fact that only approximately half the usual amount of complement is necessary for 50% hemolysis, with 4% carbowax-4000 present, may be of practical importance for complement-fixation tests, a possibility which is being investigated. The other polyethylene glycols are being tested for similar activity, and a detailed study of the general reaction described in this paper is under way. At present no explanation of the mechanism of the enhanced hemolytic activity of complement in the presence of polyethylene glycols can be offered, though it is perhaps worthwhile to speculate on a possible relation between the fat-solvent nature of these compounds and the lipoidal components of the erythrocytic membrane.

The author wishes to acknowledge the technical assistance of Miss Dolores Smith.

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TABLE I.

Comparison of Reciprocals of Dilutions of Complement Calculated to Give a Required Per Cent Hemolysis in Presence and Absence of 4% Carbawax-4000.

% hemolysis	Reciprocal of dilution of complement	
	Without carbawax	With carbawax
10	2000	3750
20	1710	3260
30	1525	2960
40	1390	2720
50	1270	2510
60	1165	2330
70	1065	2140
80	950	1950

pigment cells it is not essential to destroy or completely remove the neural portion of the retina. If a segment of the fully differentiated or regenerating neural retina becomes detached as an elevated fold it may survive. In the arch beneath it the retinal pigment cells immediately give rise to a secondary neural retina.

In 59 experiments segments of the eye wall containing the retinal pigment layer were im-

planted in the vitreous chamber of recipient eyes from which the lens had been removed. They also showed the development of neural retina from the pigment layer. It is therefore concluded that the regenerating neural portion of the retina in the adult urodele eye has its only source of origin from the cells of the retinal pigment layer.

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### Enhancement of Hemolytic Activity of Complement by Polyethylene Glycols.\* (17440)

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In the course of an investigation designed to test the ability of various substances to prevent the deterioration of complement, it was found that certain of the polyethylene glycols greatly enhance the hemolytic activity of complement. The polyethylene glycols, a series of polymers with the general formula  $\text{HOCH}_2(\text{CH}_2\text{OCH}_2)_x\text{CH}_2\text{OH}$ , are soluble in water and in many of the aromatic hydrocarbons. The compounds with average molecular weight up to 700 are liquids; those with average molecular weight above 1000 are wax-like solids, sold under the trade name "Carbowax".<sup>†</sup> This paper constitutes a preliminary report on the effect of the polyethylene glycol carbowax-4000 on the hemolytic activity of complement, as determined by complement titrations in the presence and absence of this compound.

**Procedure.** Complement titrations were carried out according to the 50% hemolysis end-point method developed by Mayer *et al.*,<sup>1</sup> and Kent *et al.*<sup>2</sup> The reaction mixtures, 20

ml in volume, had the following composition: 1) 1 part complement, the dilution so adjusted as to yield final dilution values ranging from 1:3300 to 1:950; 2) 1 part standardized suspension of sensitized erythrocytes; 3) either 2 parts of buffered saline,<sup>3</sup> or 2 parts of an 8% solution of carbowax-4000 in buffered saline. The sensitized erythrocytes were well mixed with either buffered saline or carbowax-4000 solution, and then the complement added dropwise with constant agitation. All components were kept in ice-water until time of incubation. The reaction mixtures thus prepared were incubated at 37°C for 60 minutes, with frequent agitation. After centrifugation of the mixtures, readings of per cent hemolysis in the supernates were made at 5500 Å in a Coleman spectrophotometer, an optical density of .500 representing complete hemolysis. Titrations were carried out in duplicate. Controls were set up of 1) sensitized erythrocytes and carbowax-4000, 2) unsensitized erythrocytes and carbowax-4000, and 3) complement, unsensitized erythrocytes and carbowax-4000.

**Results and discussion.** None of the control tests showed any hemolysis. The results of the titrations are shown in Fig. 1, in which

\* This work was supported by a grant from the National Tuberculosis Association.

† Carbon and Carbide Chemicals Corporation, New York.

<sup>1</sup> Mayer, M. M., Eaton, B. B., and Heidelberger, M., *J. Immunol.*, 1946, 53, 31.

<sup>2</sup> Kent, J. F., Bukantz, S. C., and Rein, C. R., *J. Immunol.*, 1946, 53, 37.

<sup>3</sup> Mayer, M. M., Osler, A. G., Bier, O. G., and Heidelberger, M., *J. Exp. Med.*, 1946, 84, 535.

TABLE I.  
 Effect of Methionine Deficiency Upon Enzyme Activity in Rat Liver.

Enzyme	Ration	No. of animals	Activity based on wet liver	Activity based on liver N
Xanthine oxidase	Basal	3	0*	0*
		3	0	0
	Basal + methionine	4	141	4150
		3	134	4100
		2	137	4120
Succinic dehydrogenase	Basal	3	14.8†	500†
		3	12.5	540
	Basal + methionine	4	18.0	550
		3	18.6	560
		2	18.6	580
Endogenous respiration	Basal	3	1.06†	35.0†
		3	1.07	45.0
	Basal + methionine	4	1.51	44.5
		3	1.48	45.6
		2	1.56	48.5

\* Units =  $\mu$  10<sub>2</sub> per hr per g wet liver.

† Units =  $\mu$  10<sub>2</sub> per hr per mg wet liver.

liver from the animals sacrificed at one time were pooled in 5 volumes of ice-cold 0.039 M sodium potassium phosphate buffer, homogenized for 3 minutes, and strained through gauze. This homogenate was used in the xanthine oxidase determinations according to the method of Axelrod and Elvehjem.<sup>5</sup> A portion of the homogenate was diluted with buffer to give a 10% homogenate, which was employed for measuring succinic dehydrogenase according to the method of Schneider and Potter.<sup>6</sup> Both enzymes were assayed using a Warburg bath maintained at 30°C. Aliquots of each homogenate were taken for nitrogen determinations in triplicate using a microKjeldahl procedure.

**Results.** The enzyme values as presented in Table I are based both upon liver wet weight and liver nitrogen. Since enzymes are protein in nature, loss or gain of enzyme activity as compared to general liver protein can be obtained by basing enzyme activity upon liver nitrogen. This also negates the effects of dilution of liver by glycogen or lipid. Endogenous respiration of the liver homogenates was calculated from the first 10-minute period

of oxidation before xanthine was added to the flasks for measuring xanthine oxidase activity. Endogenous respiration is reported since it is believed to be a measure of general liver metabolic rate.

As shown in the table a methionine deficiency induces a significant decrease in all 3 of the activities measured when activity is based upon wet weight of the liver. It was observed that the livers of the methionine deficient animals were somewhat infiltrated with fat, which probably accounts for the more noticeable decrease of wet weight enzyme activity in the amino acid deficient rats. The comparative loss of enzyme activity with general liver protein is not so pronounced in the case of succinic dehydrogenase activity. It appears that activity of this enzyme, which according to Schneider<sup>7</sup> is centered mainly in the mitochondrial elements of the liver cell, is held almost as tenaciously as other liver protein.

The striking disappearance of xanthine oxidase activity during a methionine deficiency is difficult to explain. Seifter *et al.*<sup>1</sup> have reported that during protein starvation the riboflavin content of the liver is lowered. This would not, however, explain such a marked loss in flavin enzyme activity. It ap-

<sup>5</sup> Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1941, 140, 725.

<sup>6</sup> Umbreit, *et al.*, *Manometric techniques and related methods for the study of tissue metabolism*, 1945.

<sup>7</sup> Schneider, W. C., *J. Biol. Chem.*, 1946, 165, 585.



# Effect of Methionine Deficiency upon Enzyme Activity in the Rat.\* (17441)

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The effects of low protein intake upon enzyme activity in the rat have been studied recently by several workers. Seifter *et al.*<sup>1</sup> have shown that during complete dietary protein deprivation the loss of D-amino acid oxidase and arginase activity in rat liver occurs faster than loss of general liver protein. Westerfeld and Richert<sup>2</sup> have reported that xanthine oxidase activity is decreased in rats fed low protein diets. We<sup>3</sup> have observed that liver xanthine oxidase activity is very sensitive to subtle changes in nitrogen intake. Adult rats fed a diet completely adequate for maintenance may show a liver xanthine oxidase content far below normal. Moreover it was shown that addition of 0.25% DL-methionine to the diet increased xanthine oxidase activity to normal.

The present paper is a preliminary report concerning the effects of a true amino acid deficiency upon liver enzyme activity. Because of the observed strongly limiting effects of methionine upon liver xanthine oxidase activity, this amino acid was chosen for the first study. Subsequently we expect to report the effect of other amino acid deficiencies upon liver enzyme activity.

**Experimental.** Eighteen male, weanling albino rats of the Holtzmann strain were placed on a good stock ration for 10 days. They were then given, for one week, a purified ration reported by Ramasarma *et al.*<sup>4</sup> to be the best

for growth of young rats. Briefly, this ration consisted of a purified amino acid mix (16%), salts, the known vitamins, corn oil, and sucrose. The animals were fed and watered *ad libitum* during the 7-day period. At the end of that time the animals were divided into 2 groups of 9 animals each. One group was given the complete ration, and the other group was fed the complete ration less methionine. In order to prevent poor food intake due to the methionine-deficiency both groups of animals during the experimental period were force fed in 3 daily portions an amount of the ration equal to the average intake of the animals during the *ad libitum* feeding period. The rations were taken up in distilled water to make a thick suspension and were fed by stomach tube.

After 14 days of the force-feeding period a methionine deficiency was distinctly evident in the animals receiving the methionine deficient diet as evidenced by bleeding of the feet and mouth and general loss of weight. During this period 3 animals of the group receiving the methionine-deficient diet died. The animals receiving the complete diet gained weight throughout the period and showed no detrimental signs because of the regimen.

When the enzyme experiments were begun it was thought best to sacrifice the animals as quickly as possible in order to prevent variations due to different lengths of time on the diets. For this reason 3 animals from the methionine-deficient group were sacrificed on one day; the remaining 3, the next. The animals receiving the complete ration were sacrificed in the following order on consecutive days: 4 animals, 3 animals and 2 animals.

The animals were killed by decapitation and the livers immediately removed. A portion of each liver was quickly weighed out on a delicate torsion balance. The portions of

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<sup>1</sup> Seifter, S., Harkness, D. M., Rubin, L., and Muntwyler, E., *J. Biol. Chem.*, 1948, **176**, 1371.

<sup>2</sup> Westerfeld, W. W., and Richert, D. A., *Fed. Proc.*, 1949, **8**, 265.

<sup>3</sup> Williams, J. N., Jr., and Elvehjem, C. A., *J. Biol. Chem.*, in press.

<sup>4</sup> Ramasarma, G. B., Henderson, L. M., and Elvehjem, C. A., *J. Nutrition*, 1949, **38**, 177.

TABLE I.

Effect of Vitamin B<sub>12</sub> Concentrate on Hepatic Changes of Rats Fed High-Fat Diet for 29 Days.

No. of rats	Avg wt change, g	Avg liver wt, g	Avg liver wt g/100 g rat	No. animals with hepatic fatty changes*			Avg liver fat, %
				0	1+·2+	3+·4+	
High fat diet, untreated							
6	55 ± 6.12	17.1 ± 1.25	8.1 ± 0.47	0	0	6	31.5 ± 1.18
High fat diet, + B <sub>12</sub> conc. $\approx$ 0.2 $\mu$ g B <sub>12</sub> 3 times a wk							
5	48 ± 8.13	10.4 ± 0.66	5.1 ± 0.17	1	4	0	16.3 ± 1.47

\* Grading of fatty change.

0 = None or only a rare large droplet.

1+ = Few large droplets in each lobule.

2+ = More than 1+, but less than half of lobule involved.

3+ = Over half of lobule involved, but some cells fat-free.

4+ = Almost no fat-free cells.

TABLE II.

Effect of Vitamin B<sub>12</sub> Concentrate on Hepatic Changes of Rats Fed High-Fat Diet for 66 Days

No. of rats	Avg wt change, g	Avg liver wt, g	Avg liver wt g/100 g rat	No. animals with hepatic fatty changes*			Avg liver fat, %
				0	1+·2+	3+·4+	
Normal diet, control							
4	160 ± 3.14	12.1 ± 1.13	4.0 ± 0.37	3	1	0	5.6 ± 1.08
High fat diet, untreated							
8	43 ± 9.67	15.7 ± 1.07	8.5 ± 0.62	0	1	7	24.6 ± 0.59
High fat diet, + B <sub>12</sub> conc. $\approx$ 1.0 $\mu$ g B <sub>12</sub> 3 times a wk							
10	84.6 ± 8.44	10.7 ± 0.71	4.7 ± 0.22	8	1	1	7.8 ± 0.89

\* Fatty changes graded as in Table I.

in untreated animals to 16.3% in treated rats (Table I). Histological examination also showed a partial effect of the treatment. The vitamin B<sub>12</sub> concentrate also prevented the increase in liver weight that usually occurs in untreated rats fed a high-fat diet. There was no significant difference in the gain in weight between the two groups of animals.

Treatment with a higher amount of vitamin B<sub>12</sub> concentrate (1  $\mu$ g of vitamin B<sub>12</sub> 3 times a week) over a period of 66 days demonstrated a marked lipotropic activity of the concentrate. The fat content of the liver of the treated animals was not significantly different from the amount present in rats fed a normal diet (Table II). Histological examinations of the liver also demonstrated the lipotropic activity of the vitamin B<sub>12</sub> concentrates. The weight of the liver was similarly affected by the treatment and was in the range of the normal controls. Further-

more, there was a significantly greater weight gain in the treated group as compared with the animals fed a high-fat diet alone.

**Discussion.** A vitamin B<sub>12</sub> concentrate administered over a period of 29 and 66 days to rats receiving a high-fat diet showed a significant lipotropic effect. This effect is comparable to the previous results obtained with liver extract.<sup>1,2</sup> Both vitamin B<sub>12</sub> concentrate and liver extract contained a small amount of choline and methionine. An injection of vitamin B<sub>12</sub> concentrate, containing 1 gamma of vitamin B<sub>12</sub>, supplied only 0.5 mg of choline, 0.19 mg of methionine, and 0.06  $\mu$ g of folic acid. Based on 3 injections of the concentrate per week, each rat received only 0.21 mg of choline and 0.08 mg of methionine per day. These amounts of choline and methionine *per se* are ineffective in preventing the dietary induced fatty livers. The rats injected with 0.2  $\mu$ g of

pears that the extreme lability of xanthine oxidase activity is due to several unknown factors. It is difficult to believe that activity of this enzyme is lost because of its lack of necessity to the animal since purine metabolism is believed to be very important in animal functions.

As shown in the table, general endogenous respiration of the livers of the animals receiving the complete ration and those receiving the methionine deficient ration are essentially the same. Stare and Elvehjem<sup>8</sup> had reported that endogenous respiration of chick and rat tissue is practically unaffected by various vitamin deficiencies. It has been recently re-

<sup>8</sup> Stare, F. J., and Elvehjem, C. A., *Am. J. Physiol.*, 1933, **105**, 655.

ported<sup>9</sup> that endogenous respiration of chick liver is unaffected by folic acid or vitamin B<sub>12</sub> deficiencies. It thus appears that while dietary conditions have little effect upon the total endogenous metabolism of the liver individual enzyme activities may be varied significantly by the diet.

*Summary.* It has been observed that a methionine deficiency in the rat reduces liver succinic dehydrogenase activity slightly, completely reduces liver xanthine oxidase activity, and has practically no effect upon endogenous respiration of liver tissue *in vitro*.

<sup>9</sup> Williams, J. N., Jr., Nichol, C. A., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, **180**, 689.

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## Lipotropic Effects of Vitamin B<sub>12</sub> Concentrate. (17442)

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It has recently been reported that crude liver extract exerts a lipotropic effect in rats with dietary induced liver injury.<sup>1</sup> The effect of the liver extract was not due to stimulation of the appetite with a resultant increase in protein intake, nor did the lipotropic effect seem to be related to the choline content of the liver extract. It was also demonstrated that other supplements, with a higher choline content than that of liver extract, exert a lesser degree of lipotropic activity when compared with liver extract.<sup>2</sup> In the present studies the lipotropic effect of a vitamin B<sub>12</sub> concentrate was determined in rats fed a high-fat diet.

*Methods.* Male rats of the Sprague-Dawley strain, weighing between 115 and 149 grams were used. The high-fat diet (51% lard) and the control diet (6% lard) as recently described,<sup>3</sup> were fed *ad libitum* for the

period listed in Tables I and II. When the animals were placed on the synthetic diets injections of vitamin B<sub>12</sub> concentrate were begun and were administered subcutaneously 3 times a week.\* At the end of the experiment sections were taken from the left lobe of the liver and stained with hematoxylin and eosin. Frozen sections were also made and stained with Sudan III. The remainder of the liver was analyzed for total fat.<sup>4</sup>

*Results.* In the first study treatment with a vitamin B<sub>12</sub> concentrate, in doses of 0.2 µg of vitamin B<sub>12</sub> 3 times a week, reduced the average fat content of the liver from 31.5%

<sup>3</sup> Hall, C. A., and Drill, V. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 202.

\* The Oleum Percormorphum used in the diet was kindly supplied by Mead Johnson and Company.

The vitamin B<sub>12</sub> concentrate was supplied by Dr. T. H. Jukes of Lederle Laboratories Division of the American Cyanamid Company.

<sup>4</sup> Outhouse, E. L., and Forbes, J. C., *J. Lab. and Clin. Med.*, 1939, **25**, 1157.

<sup>1</sup> Hall, C. A., and Drill, V. A., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 3.

<sup>2</sup> Drill, V. A., and Hall, C. A., *Am. J. Med. Sci.*, in press.

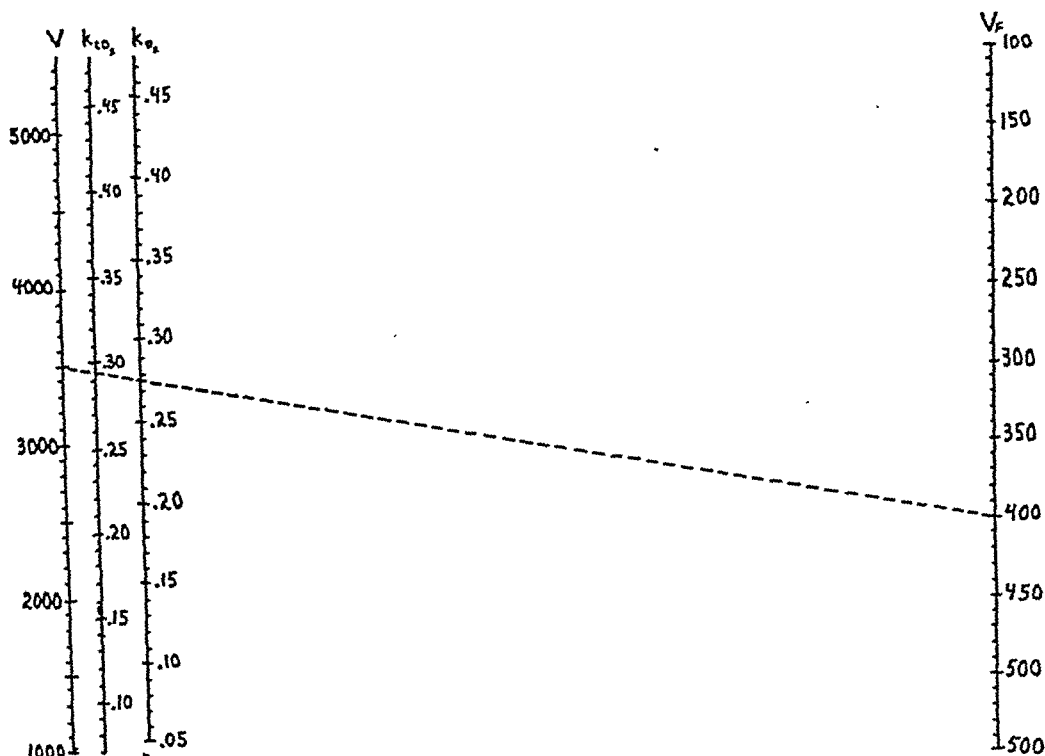


Fig. 2.  
Nomogram for determining  $k_{O_2}$  and  $k_{CO_2}$  values for micro respiration vessels.

fill the vessel and upper part of the manometer down to the 75 mark to be used as zero with one filling of mercury. This technic avoids the second stage of filling from a scratch mark to the zero with its possible errors.

In operation, the movement of fluid in the open limb of the manometer is quite rapid because of the small gas volume in the vessel for expansion or contraction. Tipping over substrate from the side-bulb requires a scrupulously clean glass surface, lest a grease layer accentuate the difficulty of pouring from such a small cup. Volumes of solution which have been found convenient to use are 300  $\mu$ l of buffered medium, 50  $\mu$ l of alkali (usually 10% KOH) in the center well and 50  $\mu$ l of substrate solution in the side-bulb. For complete  $CO_2$  and  $O_2$  determinations, 75  $\mu$ l of  $Ba(OH)_2$  may be placed in the side-arm and 100  $\mu$ l of HCl in the center well. These small volumes of fluid may be introduced into the desired parts of the vessel by means of a tuberculin syringe and needle, Kirk micro-pipette and delivering de-

vice, or glass Mohr pipette with drawn-out tip.

A nomogram constructed according to Dickens<sup>4</sup> is presented in Fig. 2 to facilitate obtaining vessel constants at 37°C for vessels from 1 to 5.5 ml in volume. It should be emphasized that V is the total capacity of vessel and manometer to the midway mark used as the zero for the constant volume readings. In the example shown, the total volume of vessel and manometer limb is 3500  $\mu$ l and the fluid volume in the vessel is 400  $\mu$ l. The  $k_{O_2}$  for this experiment is 0.277 and the  $k_{CO_2}$  is 0.294. Thus the change in manometer reading due to gaseous exchange would be approximately 3.5 times as great as the actual volume changes.

Table I lists  $Q_{O_2}$  comparisons for several rat tissues obtained in this laboratory between these vessels and the conventional 17 ml size. The metabolic activities correspond closely. The sensitivity difference is about the 4- to

<sup>4</sup> Dickens, F., *Biochem. J.*, 1945, 39, 427.

vitamin B<sub>12</sub> received only one-fifth of the amount of these added factors. The nature of the lipotropic action of the vitamin B<sub>12</sub> concentrate is not known. It is possible that the effect of vitamin B<sub>12</sub> and choline are interrelated, particularly as it has been reported that dietary choline has a specific sparing action on vitamin B<sub>12</sub> as measured by the growth of the chick.<sup>5</sup> Further studies on the possible lipotropic action of crystalline vitamin B<sub>12</sub> are in progress.

**Conclusion.** A concentrate of vitamin B<sub>12</sub> exerted a marked lipotropic effect when injected into rats receiving a high-fat diet. This lipotropic effect was not due to the small amount of choline present in the concentrate.

<sup>5</sup> Schaefer, A. E., Salmon, W. D., and Strength, D. R., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 202.

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### A Micro Version of the Barcroft-Warburg Tissue Metabolism Apparatus.\* (17443)

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Often it is desirable to measure oxygen consumption or other gaseous exchange of amounts of tissue too small to yield adequate changes in the usual 15-17 ml Warburg vessel. Several different types of apparatus have been constructed for this purpose: Warburg mentioned one of 4 ml capacity,<sup>1</sup> the Fenn type<sup>2</sup> is well known, and in recent years more diversified models have appeared (cf. evaluation by Tobias<sup>3</sup>).

A miniature form of the standard Warburg type, shown in Fig. 1, was designed of 2.5 to 3.3 ml capacity which has proved so convenient that this brief report is being made to note its availability.<sup>†</sup> The actual size is about 20 mm bottom diameter and 35 mm height, with the main vessel orifice being standard taper 14/20 and the side-bulb opening 6/15. A lip is placed on the center well, facing the side-bulb, for ease in pouring if the

barium hydroxide-hydrochloric acid procedure is used for measurement of CO<sub>2</sub> production in the same vessels in which O<sub>2</sub> consumption is determined. Four hooks, instead of the conventional two, are routinely placed on all of our flasks and manometers in order to avoid the need for repairs when one hook is accidentally knocked off.

The manometer has a graduated portion 150 mm long, made from tubing about 1 mm inner diameter, to increase the total capacity as little as possible. Calibration is carried out by the use of mercury, although it is desirable to

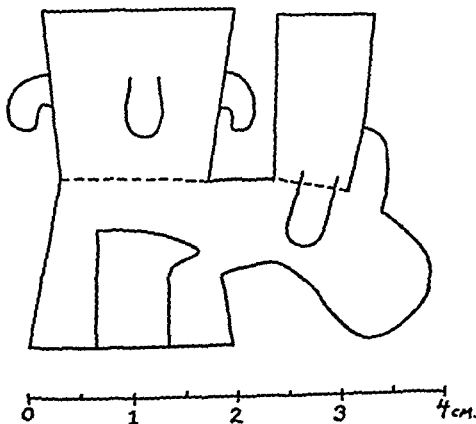


FIG. 1.  
Diagram of micro respiration vessel.

\* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships, National Institutes of Health, U. S. Public Health Service.

<sup>1</sup> Warburg, O., *Biochem. Z.*, 1923, **142**, 317.

<sup>2</sup> Fenn, W. O., *Am. J. Physiol.*, 1928, **80**, 327.

<sup>3</sup> Tobias, J. M., *Physiol. Rev.*, 1943, **23**, 51.

<sup>†</sup> The author wishes to thank the E. Machlett Company, 220 East 23rd St., New York City, for its cooperation in designing and fabricating these vessels.

TABLE II.  
Hematologic Data on Cases Treated for More than One Year.

	Avg before treatment	Avg after treatment	Increased cases	Decreased cases
Hb.	12.1 g	12.2 g	24	21
R.B.C.	4,540,000	4,480,000	17	28
W.B.C.	7,100	6,900		

acid administration to patients having macrocytic anemia, a long-term study in nonpernicious anemia persons was instituted.

*Methods.* Seventy individuals were studied for periods of from 5 to 13 months each, for a total of 768 patient months. Forty-five of the 70 were followed for more than one year. The cases were selected from hospital and out-patient population. Patients suffering from the following diseases were included, such as: rheumatoid arthritis, hypertension, rectal polyp, epididymitis, alcohol neuropathy, lues, tracheitis, neurasthenia, bronchiectasis, cholecystitis, bronchial asthma, osteoarthritis, Beck's sarcoid, cretinism and arteriosclerosis. The criteria used in selection were availability for follow-up and ability to cooperate. To reduce variables, no patients with pernicious anemia or diabetes mellitus were accepted. No attempt was made to alter general disease, as regards diet and medication. Table I shows age and sex distribution of the 45 cases studied for more than one year. Each person was given 20 mg of folic acid daily, orally, in the form of one tablet. During the period of study some patients improved from their primary disease and were discharged. This accounts for the variability in period of survey. At monthly intervals all cases were checked for hematologic data and neurologic status, which included a complete blood count and hemoglobin, gait, tendon reflexes, touch, vibration, and temperature sense, Romberg

sign and muscle grip.

*Results.* There were no changes noted during the period of study in the group of 70 patients. No patient developed any subjective or objective sign of combined sclerosis or peripheral neuritis. Hematologic data are given for the sake of completeness. Table II shows averages of hemoglobin, red cell count and white cell count for the 45 patients studied over one year. No interpretation can be offered since diet, medication and primary disease variables must be as significant as folic acid administration.

*Comment.* It is known from several studies<sup>2-6,8-11</sup> that folic acid does not protect the pernicious anemia patient from combined sclerosis. Other reports<sup>1,7</sup> have shown the appearance of combined sclerosis symptoms in patients with macrocytic anemia of gastrointestinal origin on folic acid therapy. No reports have demonstrated appearance of neurologic disease in non-macrocytic anemia patients taking folic acid. This long-term study, constituting 768 patient-months on 20 mg daily dosage of folic acid showed no neurologic toxicity. Most patients taking the drug reported a feeling of well-being. The blood counts in these non-macrocytic anemia patients were not significantly changed.

*Summary.* A study of long-term folic acid administration in 70 non-macrocytic anemia patients was made. Signs related to sub-acute combined sclerosis or peripheral neuritis did not develop in any of the group.

<sup>11</sup> Wagley, P. F., *New England J. of Med.*, 1948, 238, 11.

TABLE I.  
Comparison of Amounts of Rat Tissue and  $Q_{O_2}$  Values in Standard and in Micro Vessels.

Tissue	Micro vessel		Macro vessel	
	Tissue wt, mg*	$Q_{O_2}$ †	Tissue wt, mg*	$Q_{O_2}$ †
Diaphragm	40	0.98	150	1.02
Heart	40	0.76		
Skeletal muscle	60	0.57	300	0.45
Kidney	15	3.32	80	3.25
Liver	40	1.29	200	1.34
Thyroid	20	0.95		

\* Wet weights are used throughout.

† cu mm  $O_2$ /mg wet wt/hr.

5-fold one would anticipate from the difference in constants. There is little reason for using the micro-vessels for a tissue as metabolically active as kidney, because of the great care required in weighing the approximately 15 mg samples. The real value is in such cases as skeletal muscle, with a low rate of metabolism, or thyroid gland, with so little total tissue available.

*Summary.* A micro modification of the standard Warburg vessel for the study of gaseous exchange of tissues has been de-

scribed. As would be anticipated from the decrease in size from 17 ml to about 3 ml, the amounts of tissue required for satisfactory results can be reduced to about one-fifth those usually used.

An advantage over other more sensitive apparatus is that the manometer supports are interchangeable with the standard Barcroft-Warburg supports, so the same water-bath and shaking device can be used for both.

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### Administration of Folic Acid to Patients Without Anemia.\* (17444)

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In view of reports<sup>1-11</sup> showing the appearance of neurologic disease following folic

\* The folic acid used in this study was furnished by the Lederle Laboratories Division, American Cyanamid Company.

<sup>1</sup> Davidson, L. S. P., and Girdwood, R. H., *Lancet*, 1948, **254**, 360.

<sup>2</sup> Hall, B. E., and Watkins, C. H., *J. Lab. and Clin. Med.*, 1947, **32**, 622.

<sup>3</sup> Heinle, R. W., and Welch, A. D., *J.A.M.A.*, 1947, **133**, 739.

<sup>4</sup> Jacobson, S. D., Berman, L., Axelrod, A. R., and vander Heide, E. C., *J.A.M.A.*, 1948, **137**, 825.

<sup>5</sup> Meyer, L. M., *Bull. N. Y. Acad. Med.*, 1946, **22**, 484.

<sup>6</sup> Meyer, L. M., *Blood*, 1947, **2**, 50.

<sup>7</sup> Meyer, L. M., *Am. J. Clin. Path.*, 1948, **18**, 811.

TABLE I.  
Distribution of Cases Treated for More Than One Year. ♂ 24 ♀ 21.

Age	No.
10-19	1
20-29	3
30-39	8
40-49	8
50-59	8
60-69	9
70-79	7
80-89	1

<sup>8</sup> Ross, J. F., Belding, H., and Paegel, B. L., *Blood*, 1948, **3**, 68.

<sup>9</sup> Vilter, C. F., Vilter, R. W., and Spies, T. D., *Proc. Central Soc. Clin. Research*, 1946, **19**, 26.

<sup>10</sup> Vilter, C. F., Vilter, R. W., and Spies, T. D., *J. Lab. and Clin. Med.*, 1947, **32**, 262.

*Discussion.* The use of tungstate and sulfuric acid to prepare a protein free filtrate, while not removing the carbohydrate is a standard procedure in blood analysis work.<sup>4</sup> The fact that the blood group specific substances are carbohydrate like lends them admirably to this procedure. The method was probably never attempted before because of the nature of the reagents used since there was some doubt in our mind initially as to whether the blood group specific substance would withstand this treatment. It is not claimed that a superior product is obtained

by the Folin-Wu technic in comparison to the method of Goebel, but the procedural simplicity of the former makes it a method of choice which should be easily used when animal tissues are the source of blood group specific substances.

*Summary.* A method for preparing blood group specific substance A from Difco neopeptone by the Folin-Wu technic is described and comparisons with other preparations are made. The method is simple and the product obtained compares favorably in purity and potency with products obtained by other methods.

<sup>4</sup> Andrews, J. C., and Kyker, G. C., 1947, A Laboratory Manual of Biological Chemistry, Edwards Brothers, Inc., Ann Arbor, Mich.

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## Influence of Adrenal Cortical Steroids and Related Compounds on Sodium Metabolism.\* (17446)

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In a previous paper from this laboratory<sup>1</sup> it was demonstrated that as little as one microgram of desoxycorticosterone produced a significant retention of sodium in the adrenalectomized male rat. This communication is concerned with the study of various adrenal cortical steroids and related compound on sodium metabolism by the same technic. These steroids include 11-dehydrocorticosterone, 17-hydroxycorticosterone, corticosterone, pregnanetrione-3, 11, 20, allopregnanetriol-3 ( $\beta$ ), 17 ( $\alpha$ ), 21-one-20, alloprenanepentol-3 ( $\beta$ ), 11( $\beta$ ), 17( $\alpha$ ), 20, 21-3( $\beta$ ), 20, 21 triacetate,  $\Delta^4$ -prenenol-21-trione-3,12,20 acetate, testosterone, estradiol, and pregnanetriol-3,12,20.

*Animals, Materials, Methods.* The rats

were obtained from Carworth Farms. They were bilaterally adrenalectomized in one stage under ether anaesthesia. The animals subsisted exclusively on a chow diet both before and after adrenalectomy. When more than 24 hours elapsed between adrenalectomy and the day the experiment was run the rats were given normal saline in place of ordinary drinking water until the morning of the experiment.

The test material was injected subcutaneously in 0.25 cc of corn oil. One hour later the rats received subcutaneously, 2 cc of a solution containing sodium chloride and the radiosodium.<sup>†</sup> The dose of sodium chloride was 35  $\mu$ g per gram of body weight. The animals were placed in glass collecting cages and the urine collected for 6 hours. The urine was dried and the concentration of radiosodium in the residue determined as described previously.<sup>1</sup>

\* Supported in part by research grants from the Division of Research Grants and Fellowships of the Public Health Service, and Sharp and Dohme, Inc., Glenolden, Pa.

<sup>1</sup> Dorfman, R. I., Potts, A. M., and Feil, M. L., *Endocrinology*, 1947, 41, 464.

<sup>†</sup> The radiosodium was supplied by the Monsanto Chemical Company, through the U. S. Atomic Energy Commission.



## Simplified Technic for Preparing Blood Group Specific Substance A. (17445)

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Since the blood group specific substances are carbohydrate like in nature it seemed logical that such a simple technic as preparing a protein free filtrate by the Folin-Wu method would be an easy way to prepare blood group substance A from Difco neopeptone. As will be shown the blood group substance was not destroyed by the reagents used in the Folin-Wu adaptation. The product which is obtained compares favorably in potency and purity with preparations made with more difficult procedures.

**Materials and Methods.** The starting material for this Folin-Wu adaptation was a 5% solution of Difco neopeptone in distilled water. For every 30 ml of neopeptone solution 15 ml of 10% sodium tungstate was added, followed by the slow addition of 15 ml of 2/3 N H<sub>2</sub>SO<sub>4</sub>. After all the sulfuric acid was added the container was shaken and then left to stand for 10-15 minutes. The filtrate which contains the blood group specific substance was dialyzed against cool running water until a negative test for tungstate, sodium and sulfate was obtained. The various chemical tests such as the picric acid, Molisch, etc. were run on this dialysate (recorded under results). Two procedures were used to prepare the final product: either, (1) the dialysate was precipitated with 10 volumes of acetone, centrifuged and dried in a vacuum desiccator, or 2) dialyzed against several changes of physiological saline and stored in the liquid form. The former product was tested in inhibition studies on a dry weight basis and compared with products prepared by the method of Goebel.<sup>1</sup> It should be recalled that this investigator prepared blood group specific substance A from neopeptone by multiple precipitations with 95% alcohol, using the deproteinizing method of Sevag.<sup>2</sup> In addition to comparing the latter

product of our method with the product obtained by Goebel's method, it was compared with the blood group substance A content of a commercial product (Sharp-Dohme).

**Anti-sera.** Anti A sera for tests was prepared so as to contain as nearly as possible 8 units of isoagglutinin per unit volume.<sup>3</sup>

**Inhibition tests.** One drop of anti-A sera was added to one drop of the particular strength of the blood group specific substance. The tube was shaken and left to stand for 45 minutes at room temperature. One drop of a 2% suspension of A cells was added, the tube was shaken and left to stand for one hour. The presence or absence of agglutination was determined macroscopically. The technic is a modification of Wiener's<sup>3</sup> method for testing for secretors.

**Results.** The saline dialysate compared favorably with the commercial preparation in the inhibition tests for blood group specific substance A. The dialysate of our preparation inhibited agglutination through a titer of 256, while the commercial product inhibited agglutination through the titer of 128.

On a dry weight basis our product inhibited the agglutinating properties of 1 drop of anti-A sera when only 1 mg was present in 256 ml of physiological saline. The Goebel product which we prepared by his method gave inhibition when only 1 mg was present in 205 ml of physiological saline.

The Goebel method in our hands as well as the Folin-Wu technic gave a product free of amino acids according to the picric acid, Millons, Biuret and Hopkins-Cole tests. The commercial product gave a strongly positive Millons test. The Molisch reaction was strongly positive with all products.

<sup>2</sup> Sevag, M. C., *Biochem. Z.*, 1934, **273**, 419.

<sup>3</sup> Wiener, A. S., 1945, *Blood Groups and Transfusions*, Charles C. Thomas, Springfield, Ill.

<sup>1</sup> Goebel, W. F., *J. Exp. Med.*, 1938, **68**, 221.

TABLE III.

Influence of  $\Delta^4$ -Pregnenol-21-Trione-3,12,20-21-Acetate on Excretion of Na in Adrenalectomized Rats.

Control			Experimental			
Mean B.W. g $\pm$ S.E. (No.)	Mean Na excretion % $\pm$ S.E.	Amt. $\mu$ g	Mean B.W. g $\pm$ S.E. (No.)	Mean Na excretion % $\pm$ S.E.	Change, %	t
132 $\pm$ 4 (9)	1.53 $\pm$ 0.23	100	127 $\pm$ 3 (9)	1.75 $\pm$ 0.17	+14	0.76
124 $\pm$ 3 (8)	4.30 $\pm$ 0.55	400	126 $\pm$ 5 (5)	4.21 $\pm$ 0.50	- 2	
169 $\pm$ 3 (11)	3.52 $\pm$ 0.41	1340	177 $\pm$ 4 (12)	2.36 $\pm$ 0.39	-33	2.03

TABLE IV.

Activities of Various Steroids on Na Excretion in Adrenalectomized Rats.

Compound	Dosage, $\mu$ g	Type of activity
Desoxycorticosterone	1*	Retention
Desoxycorticosterone acetate	25*	"
$\Delta^4$ -Pregnenol-21-trione-3,12,20-21-acetate	100	Negative
	400	"
	1340	Retention
17-Hydroxycorticosterone	25	Negative
	50	Increased excretion
	200	Negative (?)
11-Dehydrocorticosterone acetate	100	Negative
Pregnanetrione-3,11,20	200	"
Corticosterone	400	"
Allopregnanepentol-3( $\beta$ ), 11( $\beta$ ), 17( $\alpha$ ), 20,21-3( $\beta$ ),20,21-triacetate	800	"
Allopregnanetriol-3( $\beta$ ), 17( $\alpha$ ), 21-one-20	800	"
Testosterone	2000	"
Estradiol	2000	"
Pregnanetrione-3,12,20	2000	"

\* Lowest concentration studied.

of this adrenal cortical steroid. The influence of 17-hydroxycorticosterone on the excretion of sodium in the adrenalectomized rat is represented in Table II. At a level of 25  $\mu$ g a mean excretion of 4.88%  $\pm$  0.93% was found as compared to the control value of 4.59%  $\pm$  0.48% which represented an insignificant change of +6%. When the dose of 17-hydroxycorticosterone was increased to 50  $\mu$ g an increase in sodium excretion of +65 and +77% respectively in 2 experiments was observed. The t values for these experiments were 2.570 and 2.020. At 200  $\mu$ g a tendency toward increased excretion of sodium was observed in one experiment (+44%, t = 1.888) while in a second experiment no significant change was observed.

$\Delta^4$ -pregnenol-21-trione-3,12,20 was studied at dose levels of 100, 400, and 1340  $\mu$ g (Table

III). At a dose level of 1340  $\mu$ g a statistical significant retention of 33% (t = 2.03) was found. This is the first instance of biological activity being found for this compound. At 100 and 400  $\mu$ g dose levels no significant change in sodium excretion was observed.

All other steroids studied were inactive at the doses tested. These included: pregnanetrione-3,11,20 (200  $\mu$ g), corticosterone (400  $\mu$ g), allopregnanepentol-3( $\beta$ ), 17( $\alpha$ ), 11( $\alpha$ ), 20, 21-3( $\beta$ ), 20,21-triacetate (800  $\mu$ g), allopregnanetrione-3,12,20 (2000  $\mu$ g), testosterone (2000  $\mu$ g), and estradiol (2000  $\mu$ g).

**Discussion.** A compilation of the results is presented in Table IV. Desoxycorticosterone is by far the most active substance tested having given positive results at one microgram. The acetate produced significant retention at 25  $\mu$ g. No lower concentrations have been

TABLE I.  
Influence of 11-Dehydrocorticosterone Acetate on Excretion of Na in the Adrenalectomized Male Rat.

Control			Experimental			
Mean B.W. g $\pm$ S.E. (No.)	Mean Na excretion % $\pm$ S.E.	Amt, $\mu$ g	Mean B.W. g $\pm$ S.E. (No.)	Mean Na excretion % $\pm$ S.E.	Change, %	t
115 $\pm$ 5 (9)	4.57 $\pm$ 1.0	25	113 $\pm$ 3 (9)	5.04 $\pm$ 0.75	+10	
115 $\pm$ 5 (9)	4.57 $\pm$ 1.0	75	119 $\pm$ 3 (9)	3.39 $\pm$ 0.45	-26	1.03
115 $\pm$ 5 (9)	4.57 $\pm$ 1.0	100	122 $\pm$ 3 (9)	3.79 $\pm$ 0.54	-17	
124 $\pm$ 3 (8)	4.30 $\pm$ 0.55	100	137 $\pm$ 4 (9)	4.88 $\pm$ 0.42	+14	0.81

TABLE II.  
Influence of 17-Hydroxycorticosterone on Excretion of Na in Adrenalectomized Male Rats.

Control			Experimental			
Mean B.W. g $\pm$ S.E. (No.)	Mean Na excretion % $\pm$ S.E.	Amt, $\mu$ g	Mean B.W. g $\pm$ S.E. (No.)	Mean Na excretion % $\pm$ S.E.	Change, %	t
135 $\pm$ 3 (9)	4.59 $\pm$ 0.48	25	142 $\pm$ 5 (9)	4.88 $\pm$ 0.93	+ 6	
128 $\pm$ 4 (8)	1.48 $\pm$ 0.27	50	124 $\pm$ 3 (8)	2.44 $\pm$ 0.26	+65	2.570
127 $\pm$ 5 (9)	1.51 $\pm$ 0.25	50	120 $\pm$ 4 (9)	2.67 $\pm$ 0.52	+77	2.020
127 $\pm$ 5 (9)	1.51 $\pm$ 0.25	200	121 $\pm$ 6 (9)	2.18 $\pm$ 0.25	+44	1.88
139 $\pm$ 6 (8)	6.69 $\pm$ 0.55	200	143 $\pm$ 5 (8)	7.06 $\pm$ 1.06	+ 6	

The results were expressed as the amount of radiosodium excreted compared to the total amount of radiosodium administered in per cent. The effect was measured by comparing the mean percentage excretion of the experimental animals to the mean percentage excretion of the control animals which were run simultaneously.

Following is a list of compounds studied and their source.

Ciba Pharmaceutical Products, Inc.; Desoxycorticosterone, Desoxycorticosterone acetate, Testosterone, Estradiol.

Dr. R. D. H. Heard, McGill University;  $\Delta^4$ -pregnenol-21-trione-3, 12, 20-21-acetate The Upjohn Company; Allopregnane-3( $\beta$ ), 17( $\alpha$ ) 21-one-20, 17-Hydroxycorticosterone, Corticosterone

Dr. E. C. Kendall, Mayo Clinic; 11-Dehydrocorticosterone acetate, Corticosterone, 17-Hydroxycorticosterone

Dr. T. F. Gallagher, Memorial Hospital; Pregnanetrione-3,11,20

Dr. T. Reichstein, University of Basle; Allopregnane-3( $\beta$ ), 11( $\beta$ ), 17( $\alpha$ ), 20, 21, 3( $\beta$ ), 20, 21-triacetate

Dr. D. Prins, Cleveland Clinic; Pregnane-trione-3,12,20

**Results.** In a previous publication (Dorfman, Feil, and Potts<sup>4</sup>) it was shown that desoxycorticosterone produces a significant sodium retention in adrenalectomized male rats in amounts as low as one microgram. The desoxycorticosterone acetate produced retention at a concentration of 25  $\mu$ g but lower concentrations of the ester have not been studied.

Table I lists 4 experiments on the influence of 11-dehydrocorticosterone acetate on sodium metabolism in the adrenalectomized male rats. No significant changes in sodium excretion were found at concentrations of 25 to 100  $\mu$ g

virus content was confirmed by monkey inoculation prior to adaptation experiments.

*Lansing strain.* In view of the claim<sup>1,2</sup> that the use of the autolyzed brain tissue technic results in a shortening of the incubation period in mice inoculated with the Lansing strain virus, this strain was also included in some of the experiments.

*Mice and Cotton Rats.* White mice bred at the Michigan Department of Health (Rockland Swiss mice) were used in 9 experiments, and the CFW (Carworth Farms Webster) strain of mice in 10 experiments. Eastern cotton rats bred at the Michigan Department of Health were used.

*Preparation of the Autolyzed Normal Mouse Brain Tissue Suspensions.* The technic described by Milzer and Byrd was used. The autolysis was achieved by keeping the sacrificed mice at room temperature for 16-17 hours prior to the removal of the brains. In most cases, the temperatures were between 22°C and 25°C. However, in some experiments, temperatures lower or higher than those indicated were recorded.

In view of the high rate of bacterial contaminations, the mouse brains were emulsified in pairs and the sterility of each pool tested separately by aerobic and anaerobic culture (blood agar plates and thioglycolate broth). The suspensions were kept in the refrigerator for not more than 24 hours, and the sterile suspensions pooled. They were filtered through several thicknesses of gauze before use.

*Diluents.* In most of the experiments, either nutrient broth of the Baltimore Biological Laboratory (BBL, pH 6.83 - 7.25), or Tyrode's solution (pH 7.3 - 7.9), or both, were used as diluents. In 2 experiments, Bacto nutrient broth (pH 6.8 - 6.85), and in 2 other experiments thioglycolate sterility broth (pH 7.2 - 7.35), were used.

*Organization of the experiments.* Groups of 10 to 20 mice were inoculated with: (1) stool suspension plus ABT in nutrient broth, (2) stool suspension plus ABT in Tyrode's

solution, (3) stool suspension plus nutrient broth (no ABT), (4) stool suspension plus Tyrode's solution (no ABT), (5) ABT plus nutrient broth (no stool), (6) ABT plus Tyrode's solution (no stool). The same setup was used for infected monkey cords and the Lansing strain (mouse cords), and for other diluents. Two or, more frequently, 3 specimens were used in each experiment. The controls mentioned under (3) and (4) were included for each specimen, while those under (5) and (6) served as general controls. The same specimens were used for the experiments repeatedly up to 10 times. In the first 5 experiments, 2 groups of mice were used for each preparation, one inoculated i.c., the other i.c. and i.p. In all the other experiments, only the i.c. route was used.

The experiments with cotton rats were set up in the same way. However, smaller groups of animals (3 to 5) were used. The observation period for both mice and cotton rats was 40 days.

*Single and repeated injections, blind passages.* There were experiments with a single inoculation, and others with 2, 3, or 4 inoculations (suggestion of Dr. Milzer). In 2 experiments, there was 1 inoculation of virus-ABT mixture, followed by a series of 5 blind passages (no ABT was used for the blind passages).

*Experimental. Experiments with mice.* The Lansing strain was tested 10 times in Rockland strain mice and once in CFW mice. Nutrient broth (Bacto and BBL) and Tyrode's solution were used as diluents. In no case was any shortening of the incubation period observed.

In 12 experiments in which the stools with previously demonstrated virus content as well as the 3 monkey adapted strains were tested repeatedly, none of the mice developed any symptoms. In 2 of these experiments, the 3 monkey adapted strains were concentrated 7 times by ultracentrifugation. The final dilution of the concentrated virus (after addition of ABT) was 35%. BBL nutrient broth, Tyrode's solution and thioglycolate broth were used as diluents in these experiments.

In 4 experiments some of the mice inocu-

<sup>2</sup> Milzer, A., Byrd, C. L., and Levinson, S. D., Abstracts of Papers Presented at the 47th General Meeting of the Soc. Am. Bacteriologists, 1947, 74.

tested.  $\Delta^4$ -pregnenol-21-trione-3,12,20 acetate has been found to cause significant sodium retention at 1340  $\mu\text{g}$  and a negative effect at 400  $\mu\text{g}$ . Thus the introduction of the 12 keto group results in a compound possessing about 2 to 5% of desoxycorticosterone. 17-Hydroxycorticosterone produced an increased sodium excretion at 50  $\mu\text{g}$  but the effect was either minimized or obliterated at 200  $\mu\text{g}$ . Increased urinary excretion of sodium has been observed previously for 17-hydroxycorticosterone and 17-hydroxy-11-dehydrocorticosterone in normal dogs and rats<sup>2</sup> and in partially depancreatized rats.<sup>3</sup> Ingle and coworkers<sup>4</sup> have found that relatively large doses of ether 17-hydroxycorticosterone or 17-hydroxy-11-dehydrocorticosterone caused an immediate increase in

sodium excretion during the first 24 hours but that the sodium excretion returned to the control levels in spite of continued treatment.

**Summary.** Eleven steroid compounds have been studied as to their influence on sodium excretion in adrenalectomized rats. Desoxycorticosterone caused significant retention at one microgram, and the acetate was effective at 25 micrograms, the lowest concentration studied.  $\Delta^4$ -pregnenol-21-trione-3,12,20-21-acetate gave a significant retention at 1340 micrograms. 17-Hydroxycorticosterone caused a significant excretion of sodium.

The following steroids were found to be negative: 11-dehydrocorticosterone acetate (100  $\mu\text{g}$ ), pregnanetrione-3,11,20 (200  $\mu\text{g}$ ), corticosterone (400  $\mu\text{g}$ ), allopregnanepentol-3 ( $\beta$ ), 11( $\beta$ ), 17( $\alpha$ ), 20, 21-3( $\beta$ ), 20, 21-tri-acetate (800  $\mu\text{g}$ ), allopregnanetriol-3( $\beta$ ), 17( $\alpha$ ), 21-one-20 (800  $\mu\text{g}$ ), testosterone (2000  $\mu\text{g}$ ), estradiol (2000  $\mu\text{g}$ ), and pregnanetrione-3,12,20 (2000  $\mu\text{g}$ ).

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<sup>2</sup> Thorn, G. W., Engel, L. L., and Lewis, R. A., *Science*, 1941, 94, 348.

<sup>3</sup> Ingle, D. J., and Thorn, G. W., *Am. J. Physiol.*, 1941, 132, 670.

<sup>4</sup> Ingle, D. J., Sheppard, R., Evans, J. S., and Kuizenga, M. H., *Endocrinology*, 1945, 37, 341.

## Evaluation of Autolyzed Mouse Brain Tissue Method for Isolation and Adaptation of Poliomyelitis Virus. (17447)

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A method for isolation and adaptation of poliomyelitis virus by direct passage into mice was described by Milzer and Byrd.<sup>1</sup> The method consists in mixing suspensions of infected feces or central nervous system (CNS) material with an equal amount of a 10% suspension of autolyzed normal mouse brain tissue (ABT) and injecting the mixture intracerebrally into white mice. It is obvious that a usable method for primary isolation of poliomyelitis virus without the use of expensive monkeys would present a great advantage. The authors undertook, therefore, to verify this method and carried out a total of 19 ex-

periments with mice and 4 with cotton rats.

**Materials and Methods.** *Feces.* Five specimens were taken from poliomyelitis patients, but not tested for virus content. The presence of poliomyelitis virus in 7 other stool specimens was demonstrated by monkey inoculation prior to adaptation experiments. (The stool specimens were obtained through courtesy of Dr. Franklin H. Top, Herman Kiefer Hospital, Detroit, and Dr. Joseph L. Melnick, Yale University).

**Monkey adapted strains.** Spinal cords of monkeys infected with poliomyelitis virus strains Buffalo, Mahoney and Tennessee were obtained through courtesy of Dr. Thomas Francis, Jr., University of Michigan. The

<sup>1</sup> Milzer, A., and Byrd, C. L., *Science*, 1947, 105, 70.

dose of virus, makes this highly improbable.

**Summary and conclusions.** The effectiveness of the autolyzed normal mouse brain method for isolation and adaptation of poliomyelitis virus was investigated in 19 experiments with mice and 4 experiments with cotton rats. Three monkey adapted strains (Tennessee, Mahoney and Buffalo) and 12 stool specimens from poliomyelitis patients were used. The Lansing strain was also included in the experiments.

In the experiments with mice, 2 strains of

neurotropic viruses were isolated. However, the evidence presented suggests that the strains isolated were of mouse origin. The cotton rat experiments were entirely negative. A shortening of the incubation period in mice inoculated with the Lansing strain was not observed.

The data presented do not confirm the effectiveness of this method for the isolation or adaptation of poliomyelitis virus.

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## Actions of 2,6-Diaminopurine in Mice, Rats, and Dogs.\* (17448)

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Recent studies have shown 4-aminopteroyl-glutamic acid (4-amino-PGA) to cause changes in hematopoietic tissues and intestinal mucosa which closely resemble lesions found in folic acid-deficient mammals.<sup>1,2</sup> These findings and the fact that 4-amino-PGA and its congeners act as competitive antagonists of folic acid (PGA) in certain microorganisms<sup>3-6</sup> have led to the conception that the agents damage tissues in mammals by interference with metabolic functions of PGA.<sup>1,2,5,6</sup>

### Studies of derivatives of naturally occurring

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† Post-doctorate Fellow of the National Cancer Institute; Neale Research Pathologist, University of Adelaide, S. Australia.

<sup>1</sup> Philips, F. S., Thiersch, J. B., *J. Pharm. Exp. Therap.*, 1949, 95, 303.

<sup>2</sup> Thiersch, J. B., and Philips, F. S., *Proc. Soc. Exp. Biol. and Med.*, 1949, 71, 484.

<sup>3</sup> Seeger, D. R., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.*, 1947, 69, 2567.

<sup>4</sup> Seeger, D. R., Cosulich, D. B., Smith, J. M., Jr., and Hultquist, M. E., *J. Am. Chem. Soc.*, 1949, 71, 1753.

<sup>5</sup> Oleson, J. J., Hitchings, B. L., and Subbarow, Y., *J. Biol. Chem.*, 1948, 175, 359.

<sup>6</sup> Franklin, A. L., Belt, M., Stokstad, E. L. R., and Jukes, T. H., *J. Biol. Chem.*, 1949, 177, 621.

purines and pyrimidines have uncovered another antagonist of PGA in microorganisms,<sup>7</sup> namely, 2,6-diaminopurine, which also appears active in higher organisms. Thus 2,6-diaminopurine delays development of an experimental leukemia in mice<sup>8</sup> and inhibits estrogen-induced growth in chick oviduct.<sup>9</sup> The purine is related structurally to 4-amino-PGA insofar as both compounds are condensed ring-systems containing 2,4-diamino-pyrimidine. These observations suggested that the purine might act as an antagonist of PGA in mammals. The following studies were undertaken to determine in detail, sites of action of 2,6-diaminopurine in mice, rats and dogs and to compare the lesions obtained with those seen after administration of 4-amino-PGA.

**Procedure.** Mice, rats, and dogs of the same strains and corresponding in sex, weight, age and maintenance to animals used in previous studies with 4-amino-PGA<sup>1,2</sup> were sub-

<sup>7</sup> Hitchings, G. H., Elion, G. B., Vanderberff, H., and Falco, E. A., *J. Biol. Chem.*, 1948, 174, 765.

<sup>8</sup> Burchenal, J. H., Johnston, S. F., Burchenal, J. R., Kushida, M. N., Robinson, E., and Stock, C. C., *Proc. Soc. Exp. Biol. and Med.*, 1949, 71, 381.

<sup>9</sup> Hertz, R., and Tullner, W. M., *Science*, 1949, 109, 539.

lated with mixtures of ABT and either stool suspensions or suspensions of the strains Mahoney and Tennessee, developed partial paralysis. Other symptoms such as ruffled fur, humped back, and tremors were present in some cases and absent in others. In all these cases, the character of paralysis was quite different from that observed in mice inoculated with the Lansing strain. Bacteriological examinations of the CNS revealed bacterial infection in all these cases. Histopathological examinations revealed fibrinopurulent exudate covering brain and medulla, or diffuse infiltration of the meninges, or multiple cortical abscesses, but no lesions characteristic for poliomyelitis.\*

In one of the experiments, a control mouse inoculated with a suspension of ABT in nutrient broth (no poliomyelitis virus containing material) developed paralysis similar to that observed with the Lansing strain or with some strains of mouse encephalomyelitis virus. There was no bacterial contamination. Pathological examinations did not show any poliomyelitic lesions. The CNS tissue of this mouse was passed into other mice which, however, did not develop any symptoms.

In 2 other experiments, 2 different virus strains producing paralysis in mice were isolated. One of the strains (referred to as ABC) was isolated from the CNS of one of the control mice inoculated i.c. with a suspension of ABT in nutrient broth. The mouse developed paralysis on the 14th day after inoculation. On further mouse passages the shortest incubation period was 2 to 3 days. Several of the mice inoculated i.p. developed paralysis. The shortest incubation period by the i.p. route was 6 days. Since no material was used which could possibly contain human poliomyelitis virus, no monkey was inoculated with this virus.

Another neurotropic virus (referred to as N-C strain) was isolated under the following circumstances. A stool suspension (received through courtesy of Dr. Melnick) was mixed

with a suspension of ABT in Tyrode's solution and inoculated i.c. into mice. None of the mice developed symptoms. A few mice were sacrificed at random and serial blind passages were carried out with thioglycollate broth as diluent and without addition of ABT. At the 4th blind passage one mouse developed paralysis after an incubation period of 19 days. On repeated passages the incubation period became shorter (10 days or more). There was no difference whether or not ABT was added to the inoculum. None of the mice inoculated with this strain by the i.p. route developed symptoms. Since poliomyelitis virus containing stool material was used for the very first inoculation of mice, a Rhesus monkey was inoculated with the N-C strain. A 10% suspension of virus was used, and the monkey received 1.5 ml i.c., a total of 4 ml intranasally and a total of 16 ml i.p. The monkey did not develop paralysis, tremors, or any other symptoms. There was no rise of temperature at any time.

*Experiments with cotton rats.* All 4 experiments with cotton rats were entirely negative. Three monkey adapted strains and several of the stools with previously demonstrated virus content were used for these experiments.

*Discussion.* From the data presented in this report, based on experiments carried out with a total of over 5,000 mice and 340 cotton rats, it is clear that the authors were not able to confirm the effectiveness of the autolyzed normal mouse brain tissue method either in the primary isolation of poliomyelitis virus by direct passage into rodents, or in the adaptation of monkey adapted strains to mice or cotton rats, or in the shortening of the incubation period in mice inoculated with the Lansing strain.

In a series of 19 experiments with mice and 4 with cotton rats, 2 neurotropic viruses were isolated. It seems clear, however, that the ABC strain is of mouse origin, since no poliomyelitis virus containing material was injected in this case. The circumstances of isolation of the N-C strain were different and do not exclude the possibility of it being a strain of poliomyelitis virus. The subsequent test in a monkey, however, inoculated with a large

\* The histopathological examinations were carried out by H. E. Cope, M.D., Clinical Pathologist, Division of Laboratories, Michigan Department of Health.

TABLE II.  
Hematological Data from Rats Treated with 2,6-Diaminopurine. (No. of animals used shown in parentheses).

Group	Dosage, mg/kg/ day	No. of inj.	Day of sacrifice	Peripheral blood				Femoral marrow					
				GRCYT, 10 <sup>3</sup> /mm <sup>3</sup>	LYCYT, 10 <sup>3</sup> /mm <sup>3</sup>	Hb, g/100 cc	RETIC, %	N-GRCYT, %	EOS-GRCYT, %	MYEL, %	ERYTH, %	LYCYT, %	
Control				Mean range	5.8(11) 1.9-12.4	13.4(11) 12.1-15.5	11(6) 7-17	33(12) 10-57	6(12) 2-20	19(12) 10-30	35(12) 16-70	9(12) 3-21	
Acute	100	1	1										
		2	2										
I	20,40	12-17	17-25	Mean (8) range	3.0 0.6-4.7	13.2 7.0-15.4	1.3 0-4.9	29 8-54	15 3-26	24 14-40	23 7-35	9 2-30	
II	20,40,79	15-21	22-30	Mean (8) range	1.4 0.4-2.8	13.7 10.7-18.1	0 0	10 2-25	8 3-14	23 6-31	17 3-35	42 26-65	

\* From data published previously.<sup>1</sup>  
 EOS-GRCYT, eosinophilic granulocytes,  
 GRCYT, granulocytes,  
 LYCYT, lymphocytes,  
 MYEL, metamyelocytes, myelocytes, and myeloblasts,  
 ERYTH, nucleated erythroid cells,  
 Hb, hemoglobin,  
 N-GRCYT, neutrophilic granulocytes,  
 RETIC, reticulocytes.



TABLE I.  
Toxicity of 2,6-Diaminopurine Following Intraperitoneal Administration.

Species	No. of inj.	Dose, mg/kg/day	Mortality	Day of death					Approx. LD <sub>50</sub> † mg/kg/day
				1	2	3	4	5-14	
Mouse	1	395	12/12	10	2				170
		198	11/12		9	2			
		99	0/12						
Mouse	5*	158	12/12		5	3	4		90
		79	2/12			2			
		40	0/12						
Rat	1	316	12/12	12					100
		158	11/12	7	2	2			
		79	3/12	1		1		1	
		40	0/12						
Rat	5*	158	12/12	6	6				45
		79	12/12		7	4	1		
		40	2/12					2	

\* Administered on 5 successive days.

† Estimated on log-probability paper.

jects of the present investigation. Procedures followed herein were also similar to those described previously. The purine† was used as the lactate or hydrochloride which is more soluble than the free base and was injected at physiologically tolerable temperatures after heating to dissolve the salts.

*Course of intoxication in mice and rats.* Animals receiving large intraperitoneal doses of 2,6-diaminopurine ( $\geq 500$  mg/kg) evidenced ataxia, weakness and dyspnea within 5 to 15 minutes. The initial signs of intoxication increased progressively in severity and animals succumbed with respiratory failure within 1 to 6 hours. Single lethal doses, less than 500 mg/kg caused death usually within 24 to 48 hours (Table I). Such animals lost approximately 20% of initial weight and exhibited severe diarrhea prior to death. Rats and mice which survived single, large doses lost less than 10% of initial weight during the first 24 to 48 hours and were fully recovered before the end of the first week. Repeated

doses of diaminopurine had similar, though delayed, effects (Table I). On the basis of toxicological data the response of mice and rats to diaminopurine may be differentiated from that following 4-amino-PGA. Regardless of the dose employed, animals receiving 4-amino-PGA did not respond with early manifestations of intoxication and survived for at least 3 days after poisoning. Furthermore, repeated administration of the PGA-analog proved cumulative in effect and more toxic than injection of single doses.<sup>1</sup> Table I suggests that repeated doses of diaminopurine equivalent to large fractions of the acute LD<sub>50</sub> were tolerated without fatality.

*Lesions in rats.* Animals receiving 100 mg/kg/day, and sacrificed after 24 and 48 hours showed at autopsy distended gastrointestinal canals filled with yellow fluid. Femoral bone marrow contained purple fluid instead of a gelatinous, greyish red substance. Findings in peripheral blood and smears of femoral marrows obtained at time of sacrifice are found in Table II. The findings in peripheral blood indicated no significant change in leucocytes or reticulocytes. Hemoglobin concentrations were elevated indicating hemoconcentration. Sections of sternal and femoral marrow showed progressive depletion of total nucleated elements estimated to be about 50% after 24 hours and 80% after 48 hours. Studies of

† The authors are grateful to Dr. George H. Hitchings and his associates of the Wellcome Research Laboratories for ample supplies of 2,6-diaminopurine, and also to Dr. George B. Brown and coworkers of the Division of Protein Chemistry, Sloan-Kettering Institute, for provision of samples of the agent used in the early part of these studies.

TABLE III.  
Hematological Data from Dogs Injected Intravenously with 2, 6-Diaminopurine.

Dog	Day	Daily dosage, mg/kg	Wt, kg	Peripheral blood				Sternal bone marrow									
				IICB/T, %		RETIC, %	GRCT, 10 <sup>3</sup> /mm <sup>3</sup>	LYCT, 10 <sup>3</sup> /mm <sup>3</sup>	NCC, 10 <sup>3</sup> /mm <sup>3</sup>	N-GRCT, %	EOS-GRCT, %	MYEL, %	LYCT, %	ERYTH, %			
				%	%												
1	0	50	16.3	43.0	0	17.3	.7	170	32	2	14	8	44				
	1		14.6	53.5	0	17.6	0	36	90	1	1	7	1				
2	0	50	15.7	54.0	pr	10.6	1.6	245	63	6	18	2	11				
	1		14.7	62.5	0	16.0	.2	25	95	1	2	1	1				
3	0	40	19.2	41.5	2.2	18.4	1.0	46	46	4	23	2	25				
	2		16.8	59.0	0	5.8	3.7	72	72	2	3	14	0				
4	0	40	19.0	39.0	0	36.0	3.6	49	49	5	19	4	23				
	2		16.2	58.5	.3	38.2	0	97	97	1	pr	0	2				
5	0	20	13.6	44.0	.3	16.7	4.2	45	54	2	12	21	11				
	4		13.3	41.5	0	9.8	1.1	10	85	0	5	10	0				
6	0	20	10.6	41.0	.6	20.4	1.3	80	67	1	13	8	11				
	4		8.2	51.0	0	11.2	.3	14	87	0	8	4	1				
7	0	8	18.3	40.0	.7	9.0	1.2	65*	37	3	14	16	30				
	18	20	16.7	37.0	0	15.6	.5	80†	70	5	12	8	5				
	28		13.8	40.5	0	.1	.1	7	9	3	83	3	2				
8	0	8	19.0	48.0	.6	10.8	.8	220*	41	3	8	5	43				
	18	20	17.6	45.0	0	7.6	.2	31†	61	7	21	8	3				
	28		14.1	50.5	0	5.9	.1	15	80	5	12	2	1				

\* Value at 6 days.

† Value at 21 days.

HICVT, hematocrit.

NCC, nucleated cell count.

pr, present, less than 0.5%.

Other abbreviations as in Table II.

sections and differential counts of nucleated elements in smears of femoral contents (Table II) revealed increased proportions of lymphocytes. These were located in the blood-filled, dilated sinuses of the marrow. No foci of necrosis or degeneration were found.

Histological examination of gastrointestinal tissues from the above animals revealed a general hyperemia. Actual lesions were confined to ileum and colon. Tips of villi were distended by enlarged vessels and plasma transudate under epithelial linings. Dilated capillaries in sub-mucosa, adjacent to muscular coats and surrounding crypts, contained increased numbers of leukocytes. Migration of leukocytes into the lumen of the crypts was evident, forming plugs with desquamated epithelial cells. In crypt-epithelium mitosis was almost absent and enlargement of nucleus and cytoplasm as well as desquamation with pyknosis was observed.

Animals receiving repeated injections for 17 to 30 days are listed in Table II (group I and II). Group I received either 20 mg/kg/day during the first three weeks followed by injections of 40 mg/kg/day for the remainder of the period, or 40 mg/kg/day throughout. Group II was treated in similar fashion but, in addition, was given prior to sacrifice 2 or 3 final daily doses of 79 mg/kg. Significant weight loss was observed only in rats of group II after receiving 79 mg/kg/day.

In spite of prolonged treatment animals of Group I and II were less affected than "acute" rats. However, the effect of diaminopurine on erythropoiesis was readily discernible in animals receiving the extended treatment. Depletion of nucleated red cells in marrow and reticulocytopenia were significant (I and II, Table II). Anemia also occurred in 2 of each group. No significant alteration occurred in erythrocyte-volume or content of hemoglobin. Only 6 of 8 animals in group I revealed depletion of nucleated cells in bone marrow, approximating 50%, while reduction in group II was no greater than that found in "acute" rats. Increased proportions of lymphocytes appeared only in marrows of group II (Table II). A predominance of basophilia in nucleated red cells and hypersegmentation of polymorphonuclears were evident in mar-

rows of both groups. Gastrointestinal lesions were not found microscopically in group I and were confined to minimal epithelial changes in crypts of colon and ileum in 3 of 8 rats in Group II.

Histological examination of other organs of animals in the 3 groups described above, revealed no significant lesions but for a moderate reduction of lymphoid tissue in thymus, spleen, mesenteric nodes, and intestinal wall. Reduction of myeloid tissues in spleen was also noted in animals with depleted marrows.

*Course of intoxication in dogs.* The administration of 40 or 50 mg/kg in dogs 1 to 4 (Table III) produced an immediate, transient hyperpnea. Vomiting occurred at 3 hours and continued profusely until death. Food was refused after the first day. Hemorrhagic diarrhea appeared within 24 hours and increased in severity progressively. During the course of intoxication animals lost weight (Table III) and developed severe upsets in fluid and electrolyte excretion. Thus, dogs 3 and 4 excreted less than 500 cc/day of urine prior to treatment while during 54 hours of intoxication the former produced 8 l of fluid (urine, vomitus, and loose stool) containing 184 m eq Cl<sup>-</sup> and the latter, 5 l containing 247 m eq Cl<sup>-</sup>. Intake of water *ad libitum* was increased in proportion. Dogs 1 and 2 succumbed after 40 hours; dog 4, after 54 hours; and dog 3 was sacrificed when moribund at 54 hours.

The remaining dogs (5 to 8, Table III) receiving lower daily doses of diaminopurine evidenced fewer signs of intoxication. Dogs 6, 7, and 8 lost weight. Occasional vomiting and diarrhea without blood occurred only in the last few days prior to their sacrifice (4, 29 and 31 days respectively). Dog 5 recovered after a moderate weight-loss (Table III and Fig. 1).

The course of lethal intoxication in dogs receiving large doses of diaminopurine can be of shorter duration than in animals given supralethal doses of 4-amino-PGA. Protracted emesis is not a feature of intoxication with the latter agent. Moreover, diarrhea does not appear in dogs poisoned with 4-amino-

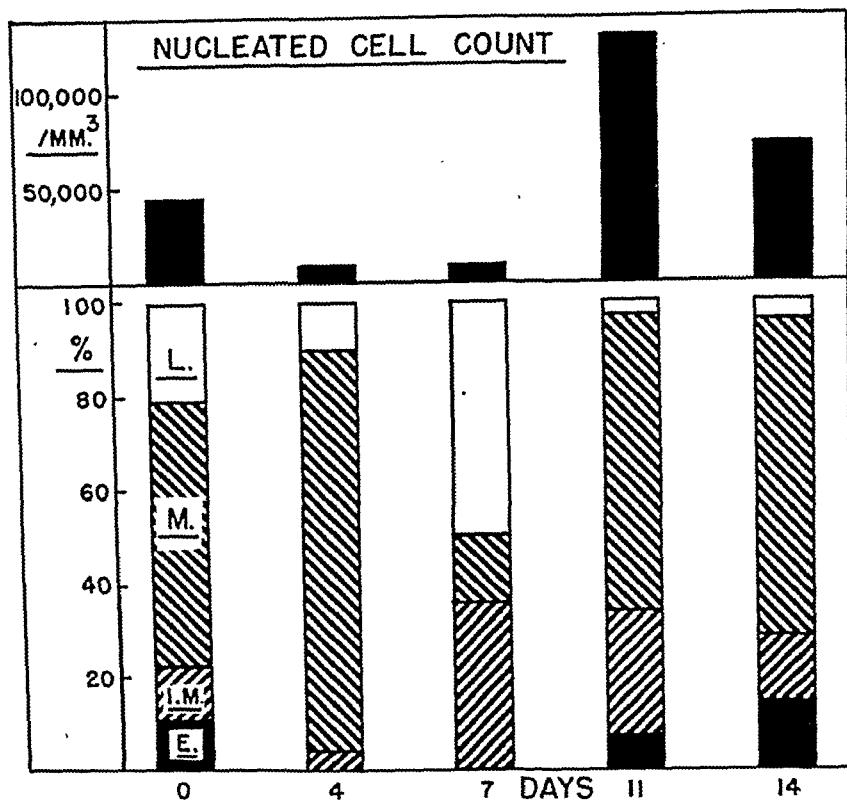


FIG. 2.

Changes in cell-count and differential count of nucleated cells from sternum of dog 5, following repeated doses of diaminopurine. L., lymphocytes; M., polymorphonuclears; IM., myeloblasts, myelocytes and metamyelocytes; E., red cells.

tract of dogs 1 to 4 revealed changes similar to those described above in rats receiving 100 mg/kg/day of diaminopurine. In addition, superficial ulcers of mucosa and extensive matting of villi occurred in ileum and colon. The remaining animals showed only edema and hyperemia of the intestinal mucosa. Changes in other tissues resembled those seen in rats.

**Discussion.** Since other purines such as caffeine<sup>10</sup> or adenine<sup>11</sup> are known to cause a variety of acute pharmacological actions, it was not unexpected that large doses of 2,6-diaminopurine should result in death within a few hours after administration. Indeed, the gross behavior of rats and mice is similar following equivalent doses of either 2,6-di-

aminopurine or adenine (6-aminopurine) greater than 500 mg/kg.<sup>12</sup> On the other hand 4-amino-PGA is inert from the standpoint of acute pharmacological effects.<sup>1,2</sup> At present it does not appear pertinent to compare diaminopurine and 4-amino-PGA on the basis of such actions which bear no known relationship to metabolite-antagonism. More appropriate is a comparison of lesions in intestines and bone marrow caused by both agents. Their effects on mucosa of ileum and colon of rats and dogs cannot be differentiated by histological studies. The depletion of hematopoietic tissues following effective doses of either agent can be of similar severity. However, certain features of the disturbance in hematopoiesis caused by 4-amino-PGA in dogs are unique

<sup>10</sup> Sollman, T., *A Manual of Pharmacology*, W. B. Saunders, Philadelphia, 1948.

<sup>11</sup> Drury, A. N., *Physiol. Rev.*, 1936, 10, 292.

<sup>12</sup> Philips, F. S., Thiersch, J. B., and Bendich, A., unpublished observations.

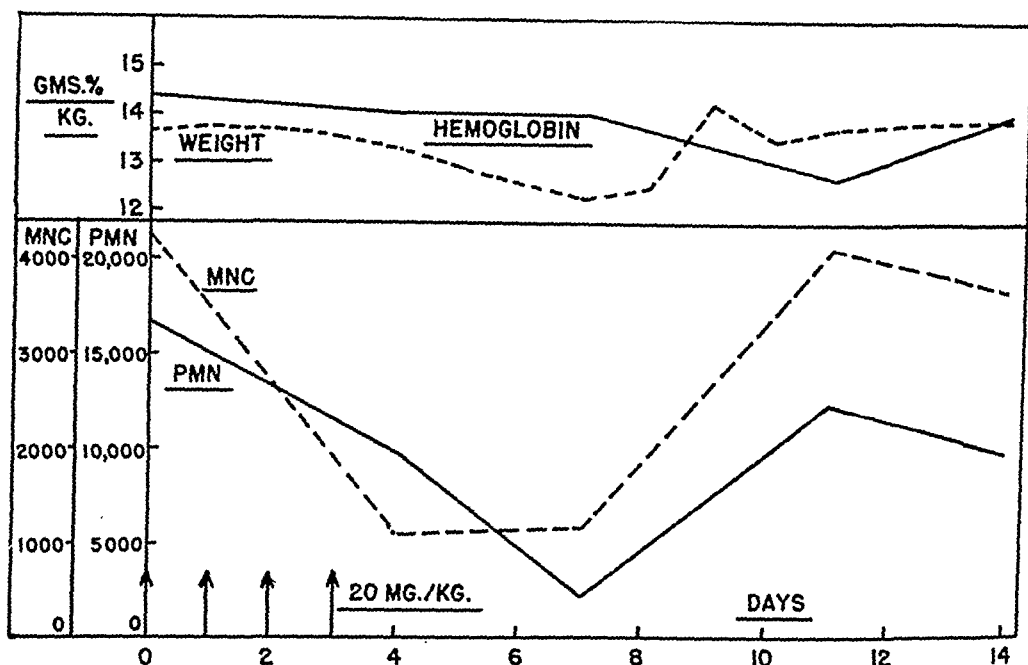


FIG. 1.

Changes in weight and peripheral blood of dog 5, following repeated doses of diaminopurine. MNC, lymphocytes and monocytes/mm<sup>3</sup>; PMN, granulocytes/mm<sup>3</sup>.

PGA until after 48 hours and death does not occur before 72 hours.<sup>2</sup>

*Lesions in dogs.* At autopsy all dogs appeared dehydrated. Bone marrow in sternum, ribs, and vertebrae appeared dry and sparse; femoral marrow, brownish and fatty. The intestinal tract of dogs 1 to 4 was edematous from duodenum to anus. Petechial hemorrhages were visible in ileum and colon and extensive around the anus. The contents of the gut were fluid and blood-stained. Dogs 6, 7 and 8 showed only edema of the intestinal mucosa.

Findings in peripheral blood are noted in Table III. Hemoconcentration developed in dogs 1 to 4 and 6 and might have been masked in dogs 7 and 8 by coexisting anemia. No changes in erythrocyte-volume or hemoglobin-content were noted. Reticulocytes and lymphocytes decreased in numbers in most animals. Granulocytopenia developed in dogs 5 to 8, which had been observed for more than 48 hours. Details of changes in peripheral blood of dog 5 are noted in Fig. 1.

Bone marrow was studied with aspirations

from sternum and both iliac crests as well as with tissue-blocks taken at autopsy from ribs and femur. Only values from sternal marrow are reported in Table III since they corresponded closely to samples from other sites. A reduction of total nucleated cells was noted in all animals when measured. This observation was confirmed by study of smears and sections which contained only scattered islands of hematopoietic tissues. The outstanding feature of the depletion was the marked reduction of erythropoietic tissues. Surviving cells of the erythroid series included the naturally occurring erythrocytes with nuclear remnants, normoblasts, and erythroblasts. Although myeloid cells shared in the general depletion, all dogs with one exception evidenced increased percentages of polymorphonuclear granulocytes with relative decreases of immature myeloid forms. The marrow of the exceptional dog, 7, contained at 28 days, a high percentage of myelocytes in association with an intercurrent infection. Details of marrow findings in dog 5 are shown in Fig. 2.

Histological study of the gastrointestinal

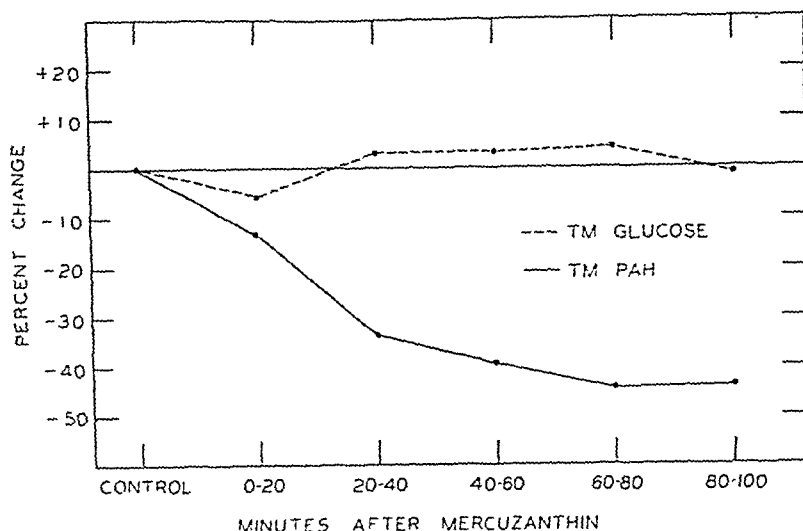


FIG. 1.

The individual points represent the mean per cent change in  $Tm_G$  of 10 subjects and in  $Tm_{PAH}$  of 6 subjects for 5 consecutive 20 minute urine collection periods following the intravenous administration of 78 mg of organically bound mercury. The control value is based on the mean of three 12 minute urine collection periods for each subject.

minute urine collection periods were obtained. The total time following injection of the mercury to the end of the last urine collection period was 100 minutes. Inulin, used in measuring the glomerular filtration rate, was determined using Harrison's modification of the method of Alving.<sup>6</sup> PAH determinations, using diluted urine and cadmium sulfate filtrates of plasma, were made by the method of Bratton and Marshall.<sup>7</sup> Glucose determinations were made according to the method of Nelson.<sup>8</sup>

**Results.** When the values for the group were averaged for each period, the maximum depression of  $Tm_{PAH}$  was 44.5% of the control value and occurred during the urine collection period from 60-80 minutes following the administration of mercuzanthin (Fig. 1). The mean  $Tm_{PAH}$  values decreased from 73.3 mg/min to 44.9 mg/min following the administration of mercury (Table I). This difference is significant ( $N = 6$ ,  $t = 11.05$ ,  $P < .01$ ). In the case of the  $Tm_G$  observations, no significant difference was noted between the

mean values of the pre- and post-mercurial observations ( $N = 10$ ,  $t = .275$ ,  $P > .70$ , Table I). The first postmercurial urine collection period (0-20 minutes) was not used in calculation of the mean for either  $Tm_G$  or  $Tm_{PAH}$  because of the possible effect of the theophylline base on the renal tubular function. The apparent decrease in  $Tm_G$  for the first post-mercurial urine collection period (0-20 minutes) is not significant.

**Comment.** The renal tubular reabsorption of glucose is generally believed to be effected through a phosphorylation mechanism.<sup>9</sup> Assuming that this is the mechanism operative in glucose reabsorption by the renal tubules, mercury must have been ineffective, at least in the amounts used in this study, in blocking the activity of this system. This belief finds an interesting parallel in the studies of Hepler and Simonds.<sup>10</sup> Using mercury chloride, these investigators demonstrated that glycosuria could not be induced in dogs unless the animals showed a 4+ albuminuria and marked renal tubular necrosis. The observa-

<sup>6</sup> Harrison, H. E., *Proc. Soc. Exp. Biol. and Med.*, 1942, 49, 111.

<sup>7</sup> Bratton, A. C. and Marshall, E. K., Jr., *J. Biol. Chem.*, 1939, 128, 537.

<sup>8</sup> Nelson, H., *J. Biol. Chem.*, 1944, 153, 375.

<sup>9</sup> Shannon, J. A., *Ann. Rev. Physiol.*, 1942, 4, 301.

<sup>10</sup> Hepler, O. E., and Simonds, J. P., *Arch. Pathol.*, 1946, 46, 398.

such as the appearance of abnormal mitosis and "nuclear explosions" in normoblasts, alteration of the nuclear pattern of mature and primitive erythroid cells and formation of cells resembling megaloblasts, and production of bizarre, giant metamyelocytes.<sup>2</sup> Similar pathological cells have not been observed in dogs receiving diaminopurine. Therefore, it cannot be inferred that the agent acts as an antagonist of PGA in the metabolism of hematopoietic tissues.

*Summary.* The administration of large doses of 2,6-diaminopurine in mice and rats

can result in death within a few hours. In dogs, large doses caused protracted vomiting, hemorrhagic diarrhea, dehydration and death within 48 hours. Doses permitting survival of rats and dogs for 24 hours or longer produced depletion of bone marrow and damage to epithelium of colon and ileum. The course of intoxication and changes in bone marrow permit a differentiation between the actions of 2,6-diaminopurine and a folic acid-antagonist like 4-aminopteroylglutamic acid.

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### Effect of Mercury on Renal Tubular Transfer of p-Aminohippurate and Glucose in Man. (17449)

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The depressant effect of intravenously administered salyrgan (mersalyl) on the maximum renal tubular secretion of p-aminohippurate ( $Tm_{PAH}$ ) in man has been demonstrated.<sup>1,2</sup> The inability of the mercurials, salyrgan and meralluride (mercuhydrin), to depress the  $Tm_{PAH}$  in the dog has also been shown.<sup>2,3</sup> That the maximum tubular reabsorption of glucose ( $Tm_G$ ) in the dog is unaffected by mercury chloride or meralluride in dosages of 4 mg of bound mercury per kilogram of body weight has been established recently.<sup>3</sup> With regard to the  $Tm_G$  in man, Weston *et al.*<sup>4</sup> have reported that the intravenous administration of 2 cc of mercuzanthin or thiomerin, "immediately after several ten minute control periods generally resulted in a 40-80% decrease."

Thus the current concept is that intraven-

ously administered mercurials depress the renal tubular transfer systems for PAH and glucose in man but leave unaffected these systems in the dog. It is the purpose of this study to re-examine the effect of a mercurial, mercuzanthin, on the  $Tm_{PAH}$  and  $Tm_G$  in man.

*Method.* Ten fasting male subjects were used in the  $Tm_G$  observations. Five of the 10 subjects used for the  $Tm_G$  observations plus an additional subject were used in the  $Tm_{PAH}$  determinations. All of the subjects were free of demonstrable renal dysfunction. The study pattern was the same for all of the subjects. Using the saturation methods outlined by Goldring and Chasis<sup>5</sup> 3 control urine collection periods, each of 12 minutes duration, were obtained. Two cc of mercuzanthin\* were injected intravenously at the end of the third control period. Immediately following the third period, 5 consecutive 20

<sup>1</sup> Brun, C., Hilden, T., and Raaschow, F., *Acta Pharm. Toxicol.*, 1947, 3, 1.

<sup>2</sup> Berliner, R. W., Kennedy, T. J., and Hilton, J. G., *Am. J. Physiol.*, 1948, 154, 537.

<sup>3</sup> Handley, C. A., Telford, J., and LaForge, M., *Proc. Soc. Exp. Biol. and Med.*, 1949, 71, 187.

<sup>4</sup> Weston, R. E., Grossman, J., Edelman, I. S., Escher, D. J. W., Leiter, L., and Hellman, L., *Fed. Proc.*, 1949, 8, 164.

<sup>5</sup> Goldring, W., and Chasis, H., *Hypertension and Hypertensive Disease*, The Commonwealth Fund, New York, 1944.

\* One cc of mercuzanthin represents 135 mg of the sodium salt of b-methoxy-hydroxy-mercuri-propylamide of cyclopentane dicarboxylic acid equivalent to 39 mg of mercury and 35 mg of anhydrous theophylline.

## Destruction of Red Blood Cells. VII. Apparent Autosensitization to Dog Red Blood Cells.\* (17450)

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In an occasional case of atypical hemolytic anemia a serum agglutinin active against the patient's own red blood cells has been found.<sup>1</sup> This may be merely the result of the presence of a serum substance with entirely fortuitous properties resembling in activity the seemingly nonspecific agglutination of red blood cells by antiserum developed in rabbits against Type 14 pneumococcus<sup>2</sup> or by certain viruses.<sup>3</sup> There are, however, two possible strictly immunological explanations for the presence of such an autoagglutinin: 1. The antibody (autoagglutinin) could be formed in response to some antigen in the red blood cell, or 2. the antibody could be formed for some other antigen but might only incidentally possess the capacity to react with a receptor in the blood cell.

That an animal can produce antibody for components of tissues of the same species has been amply demonstrated.<sup>4</sup> Hektoen and Schulhof,<sup>5</sup> after injecting rabbits intravenously with solutions of rabbit thyroglobulin, demonstrated serum precipitins for rabbit thyroglobulin in a titer as high as 1:10,000. Such sera did not react with the thyroglobulin of beef, dog, human, monkey, rat, sheep or swine. Several workers<sup>6,7</sup> have shown that formalized rabbit serum is antigenic for rabbits. Further-

more, rabbit brain extracts (containing lipoids)<sup>8</sup> and rabbit fibrinogen<sup>9</sup> stimulate the formation in rabbits of complement-fixing antibodies and precipitins. Schwentker and Comptoier<sup>10</sup> and the Caveltis<sup>11</sup> have demonstrated the formation of complement-fixing antibody for homologous renal tissue.

Such observations suggested that an animal might be sensitized experimentally to its own red blood cells. If such sensitization were demonstrated it would support the explanation that in certain instances of acquired hemolytic anemia in man the serum autoagglutinins may be formed in response to an antigen in red blood cells. Therefore, the following studies were made.

**Materials and Methods.** Four female mongrel dogs each weighing approximately 25 lb were maintained on a diet of horse meat and commercial preparations of dog food supplemented with 1.2 g of ferrous sulfate a day. Red blood cells, hemoglobin concentration, hematocrit, icterus index, and reticulocyte and white blood cells in oxalated samples of venous blood were determined by conventional methods on the average of every 10 days. Repeated determinations of osmotic and mechanical fragility of the red blood cells were made by modifications of technics previously described.<sup>12,13</sup> Serum complement titrations, complement fixation, and cold agglutinin and warm agglutinin titers were followed periodic-

\* This investigation was aided in part by a grant from the John and Mary R. Markle Foundation.

<sup>†</sup> Research Fellow of the American College of Physicians.

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TABLE I.  
Changes in  $Tm_G$  and  $Tm_{PAH}$  Following Mercury Administration.

Age, yr	$Tm_G$ , mg glucose/1.73 sq. m/min.			$Tm_{PAH}$ , mg PAH/1.73 sq. m/min.		
	Control	Post-mercury	% change	Control	Post-mercury	% change
53	296	316	+ 6.8	98.3	58.3	-40.7
57	206	191	- 7.3	—	—	—
44	254	270	+ 6.3	52.3	23.3	-55.4
59	198	200	+ 1.0	—	—	—
65	248	248	$\pm$ 0.0	50.6	24.9	-50.8
67	271	312	+15.1	—	—	—
53	377	361	- 4.2	83.2	54.3	-34.7
62	252	221	-12.3	—	—	—
61	224	267	+19.2	83.4	61.5	-26.3
48	246	238	- 3.3	—	—	—
69	—	—	—	71.9	47.2	-34.4
			Mean			
58	257.2	262.5	+ 2.1	73.3	44.9	-40.4

Control values represent the mean of 3 twelve minute urine collection periods. Post-mercury values represent the mean of 4 twenty minute urine collection periods, extending from 20 to 100 minutes following the injection of mercuzanthin.

tion that the  $Tm_{PAH}$  is diminished while the  $Tm_G$  is unchanged following the administration of mercury strongly implies that the mechanisms involved in PAH and glucose transfer are different. However, in conjunction with this belief, it is interesting to note that the simultaneous measurement of  $Tm_{PAH}$  and  $Tm_G$  in the dog results frequently in a depression of the former and occasionally in a depression of the latter.<sup>11,12</sup> In man the simultaneous determination of  $Tm_{PAH}$  and  $Tm_G$  may result in a depression of the former and an apparent increase in the latter.<sup>13</sup> At

present the observed depressions are presumed to be a result of competition for available energy between the PAH and glucose transfer systems with the latter being favored.<sup>11</sup>

**Summary.** The intravenous administration of 78 mg of organically bound mercury (2 cc mercuzanthin) causes a significant decrease in the  $Tm_{PAH}$  but no significant change in the  $Tm_G$  in man. It is concluded that under the conditions of this study mercury had no effect on the renal tubular glucose transfer system in man.

<sup>11</sup> Houck, C. R., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 398.

<sup>12</sup> Kelley, V. C., and McDonald, R. K., *Am. J. Physiol.*, 1948, **154**, 201.

<sup>13</sup> Klopp, C., Young, N. F., and Taylor, H. C., Jr., *J. Clin. Invest.*, 1945, **24**, 117.

The technical assistance of Mrs. Eleanore Roach, Mrs. Elsie Beard, Miss Margaret McCollum, Mr. Charles Punte, and Mr. Robert Faid is gratefully acknowledged.

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which the washed red blood cells were agglutinated by anti-dog serum no anemia or increase in reticulocytes or icterus index appeared. The osmotic fragility of the red blood cells remained normal. No cold or warm agglutinins were demonstrable. The complement titer of the serum (1:32) did not fall; and no serum complement-fixing antibody for the dog's own red blood cells was demonstrable. The mechanical fragility of the red blood cells ranged from 3.1 to 6.1%; values considered by Shen<sup>17</sup> to be normal for dog red blood cells.

*Summary and Conclusions.* Several attempts were made to sensitize 4 dogs to their own red blood cells. Three dogs showed no definite evidence of autosensitization. Although 2 of these 3 dogs showed occasional marked fluctuation in mechanical fragility unaccompanied by other changes during these studies this was considered to be due to tech-

nical difficulties. However, for a short period in one dog, 25 days after the injection of a modified Freund antigen, there was agglutination of washed red blood cells when they were suspended in anti-dog serum rabbit serum. During this period of apparent auto-sensitization there was no anemia, reticulocytosis or jaundice. The red blood cells showed no alteration in osmotic or mechanical fragility. There was no fall in complement titer. Cold and warm agglutinins did not appear. There was no evidence of a serum complement-fixing antibody for the dog's own red blood cells. Such apparent autosensitization supports the explanation that in certain instances of acquired hemolytic anemia in man serum autoagglutinins may be formed in response to an antigen in red blood cells.

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## The Appearance of Acetylcholine During Normal Labor. (17451)

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In our earlier studies on the acetylcholine (Ach) of the human placenta,<sup>1</sup> blood from pregnant women was not available for investigation. In consideration of the potent hydrolytic action of the blood cholinesterase, it was inferred that Ach of human placenta does not appear to reach the maternal circulation in any noticeable quantity.

Opportunity to restudy this problem has not presented itself until lately. On the basis of an analysis of 288 blood samples of 152 cases during pregnancy and at different stages of labor, our previous impression has to be modified.

Four cc of blood were obtained each time and poured into 2 volumes of 95% pure alcohol. The mixture was thoroughly ground,

diluted with 2 volumes of saline and centrifuged. The supernatant fluid was measured, and usually 0.5 cc or less was used for assay. Alcohol-free extract was also prepared by evaporating 5 cc to dryness in room (23-30°) and re-taking up with 5 cc of frog Ringer solution. The extracts were assayed on the toad's rectus test which depends on the principle of equal potentiation of Ach standard and the unknown by eserine. So if the matched ratio is identical before and after eserine, it is taken as Ach. Otherwise, Ach is considered to be nil.

Table I gives results showing a gradual appearance of Ach toward the second trimester of gestation, an apparent increase of Ach with the onset of labor, and a disappearance beginning 48 hours after labor.

The blood of 10 non-pregnant women and

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<sup>1</sup> Chang, H. C., and Wong, A., *Chin. J. Physiol.*, 1933, 7, 151.

ally according to standard technics.<sup>14,15</sup> Serum from rabbits previously injected repeatedly with normal dog serum was heated at 56°C for 30 minutes and employed in a manner similar to that described by Coombs *et al.*<sup>16</sup> Thus, equal aliquots of saline suspensions of washed dog cells were incubated for one hour at 37°C with the anti-dog serum rabbit serum, centrifuged at about 300 rpm for 2-3 minutes, resuspended by gentle agitation and examined microscopically under a cover slip for agglutination.

*Procedures and Results.* Several methods were employed. The red blood cells from 10 cc of defibrinated blood were frozen, thawed and then incubated in 4 cc of streptococcal toxin containing 400,000 skin test doses for 24 hours at 37°C. The suspension was then injected intraperitoneally into the dog from which the cells had been obtained. Fourteen such injections were made in 2 dogs at 2 day intervals. During the period of injection and for the 18 following days no significant alterations of results in the laboratory tests listed above were found.

In the study of 2 other dogs red blood cells from 10 cc of defibrinated blood were incubated 24 hours at 37°C after being inoculated with 6 to 24 hour broth culture of hemolytic *Staphylococcus aureus* (Costello strain). The blood culture was then filtered through a Seitz filter and frozen for 24-48 hours before being used. After thawing, the filtrate was injected intraperitoneally into the dog from which the cells had been obtained. To 1 dog 10 such injections were given and to another dog 11 injections at 48-72 hour intervals. During the period of injection and the following 17 days no significant alterations of results in the laboratory tests listed above occurred. One dog showed fluctuations in mechanical fragility of its red blood cells from 3.2 to 50% unassociated with other changes in the results of laboratory tests listed. As marked

fluctuations in mechanical fragility of apparently normal dog cells sporadically occur<sup>17</sup> such an isolated observation was not considered indicative of autosensitization.

Seventeen to 18 days after the previous experiments each of the 4 dogs was given a single subcutaneous injection of a modified Freund antigen<sup>18</sup> consisting of a mixture of 1 cc of its own washed, packed red blood cells, 1 cc of mineral oil<sup>†</sup>, 0.2 cc of a lanolin-like substance<sup>§</sup> and 1 mg of heat killed *Mycobacteria tuberculosis* (H37Rv strain).<sup>||</sup> Over a period of 6 weeks following the injection no changes were noted in osmotic fragility or complement titer and no development of cold or of warm agglutinins or of serum complement-fixing antibodies occurred. The washed red blood cells did not clump in anti-dog serum rabbit serum. The mechanical fragility of the cells from 2 of the dogs fluctuated in an unpredictable manner. However, in light of previous experience<sup>17</sup> this was not considered indicative of autosensitization.

Six weeks after the injection of the above described modified Freund antigen a second similar preparation was made with the red blood cells of each dog. The mixture was then heated at 57°C for 2½ hours, cooled, added to 2 cc of fresh pig serum and injected immediately subcutaneously. Again there were no changes in 3 of the dogs as tested by the laboratory procedures listed above. However, in 8 observations made from the 25th to the 35th post-injection day the washed red blood cells from the fourth dog showed definite agglutination when suspended in anti-dog serum rabbit serum. On the 36th, 37th, 38th and on subsequent post-injection days the cells were found non-agglutinable by such a procedure. During the period in

<sup>17</sup> Shen, S. C., private communication.

<sup>18</sup> Freund, J., and McDermott, K., *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 548.

<sup>†</sup> The brand of mineral oil used was Bayol F, supplied by the Standard Oil Company of New Jersey.

<sup>§</sup> The lanolin-like substance was Falba, the trade name for a preparation of Pfalz and Bauer, Inc., New York.

<sup>||</sup> Supplied through the courtesy of William Steenken, Jr.

<sup>14</sup> Boyd, W. C., *Fundamentals of Immunology*, Interscience Publishers, 1947.

<sup>15</sup> Stats, D., and Wasserman, L. R., *Medicine*, 1943, **22**, 363.

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## Probable Absence of Citrulline from Proteins. (17452)

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Several early investigators noted that carbon dioxide is one of the products of the acid hydrolysis of proteins. Dunn<sup>1</sup> found that as much as 0.81% of casein could be accounted for as carbon dioxide after 30 hours of acid hydrolysis. It has been generally supposed that this carbon dioxide is bound as a ureide in the protein molecule and the exact type of binding is uncertain. Fearon's<sup>2</sup> diacetyl monoxime test for ureide compounds is positive for many proteins. Wada<sup>3</sup> reported the isolation of citrulline from tryptic digests of casein, but this work has never been repeated. Since certain types of bacteria can convert arginine to citrulline, it is possible that Wada's citrulline may have been formed by bacterial action during the enzymatic hydrolysis.

This investigation was undertaken in order to obtain additional evidence as to the nature of the ureide groupings in proteins.

**Methods and results.** The proteins used in this study were all prepared in the laboratory. Casein was prepared according to the method of Van Slyke and Baker,<sup>4</sup> the crystalline globulins of watermelon seed and pumpkin seed by the method of Vickery,<sup>5</sup> arachin by the method of Johns and Jones,<sup>6</sup> amandin by the method of Osborne,<sup>7</sup> and myosin by the method of Dainty, Kleinzeller, *et al.*<sup>8</sup>

The 2 crystalline globulins were recrystallized twice after the initial isolation, and the

arachin and amandin were twice dissolved and precipitated by dialysis after the initial isolation. The casein and myosin were used as originally isolated.

After preparation the proteins were air dried until completely equilibrated with regard to moisture content. They were analyzed for moisture and ash, and for nitrogen by the Kjeldahl method using mercury as the catalyst. The results of these analyses are given in Table I.

These proteins gave moderate to marked positive tests for ureide groups when tested under the conditions of Archibald's<sup>9</sup> citrulline determination. After they had been hydrolyzed for 16 hours or longer with 6N hydrochloric acid, none of them gave positive tests. It was thought that this might be due to destruction of any citrulline contained in the proteins during the hydrolysis, so the destruction of citrulline alone under the conditions employed for hydrolysis of the proteins was studied. One ml samples of solutions of citrulline (about 1 mg/ml) in 6N hydrochloric acid were sealed in small glass bomb tubes and heated in the oven at 110°C. At intervals samples were removed and cooled, and their citrulline content was determined by Archibald's method. Under these conditions citrulline is surprisingly resistant to acid hydrolysis; it is destroyed by a first order reaction whose half-life is approximately 26 to 28 hours, as is seen in Fig. 1. This is not conclusive evidence for the absence of citrulline from proteins since it is well known that cystine and threonine are much more easily destroyed when in peptide combination than when free. Other experiments in which paper chromatography was employed to identify the products of hydrolysis of citrulline showed that under these conditions ornithine was the only product formed in detectable amounts.

Since Wada's isolation of citrulline was

<sup>1</sup> Dunn, M. S., *J. Am. Chem. Soc.*, 1925, **47**, 2564.

<sup>2</sup> Fearon, W. R., *Biochem. J.*, 1939, **33**, 902.

<sup>3</sup> Wada, M., *Biochem. Z.*, 1933, **257**, 1.

<sup>4</sup> Van Slyke, L. L., and Baker, J. C., *J. Biol. Chem.*, 1918, **35**, 127.

<sup>5</sup> Vickery, H. B., Smith, E. L., Hubbell, R. B., and Nolan, L. S., *J. Biol. Chem.*, 1941, **140**, 613.

<sup>6</sup> Johns, C. O., and Jones, D. B., *J. Biol. Chem.*, 1917, **28**, 77.

<sup>7</sup> Osborne, T. B., *Am. J. Physiol.*, 1907-08, **20**, 470.

<sup>8</sup> Dainty, M., Kleinzeller, A., Lawrence, A. S. C., Miall, M., Needham, J., Needham, D. M., and Shen, Shih-Chang, *J. Gen. Physiol.*, 1943-4, **27**, 355.

<sup>9</sup> Archibald, R. M., *J. Biol. Chem.*, 1944, **150**, 121.

TABLE I.  
Increase in Acetylcholine of Blood During Pregnancy.

	During pregnancy			Before onset of labor	During labor		After labor	
	1st trimester	2nd trimester	3rd trimester		Early stage	Late stage	Before 48°	After 48°
Total No. of samples	26	30	23	26	44	66	47	26
Samples without Ach: No.	25	21	17	10	8	17	34	26
%	96.2	70.0	73.9	38.5	18.2	27.8	72.3	100
Samples with Ach: No.	1	9	6	16	36	49	13	0
%	3.8	30.0	26.1	61.5	81.8	72.2	27.7	0
Range of Ach in blood, γ/cc	1.61	0.69-3.06	0.71-1.94	0.73-4.70	1.02-7.59	0.71-6.81	0.67-6.21	—
Average	—	1.871 ± 0.220	1.332 ± 0.164	2.586 ± 0.324	3.089 ± 0.286	2.387 ± 0.199	2.929 ± 0.450	—

10 male individuals showed no evidence of Ach by the same test. Trichloroacetic extract prepared according to Chang and Gaddum<sup>2</sup> gave results of the same order as that of alcohol extract.

Although alcohol has a potentiating action on Ach,<sup>3,4</sup> it did not appear to influence the results of our test under the routine precautions taken. In 25 trials to study this point, the alcoholic extracts gave an average of  $1.355 \pm 0.090$  γ/cc, while the corresponding alcohol free extracts gave an average of  $1.181 \pm 0.105$  γ/cc, the difference being  $0.174 \pm 0.139$ .

From the studies made on placental tissue and perfused placenta,<sup>5</sup> it was suggested that Ach may play a local role in the normal mechanism of labor. Any theoretical deduction made from studies on a tissue which has been discharged as being of no more use to the body is to be guarded against. By demonstrating an increased blood Ach toward term and with the onset of labor, our suggestion that Ach may play a role in the normal mechanism of labor is further strengthened.

In view of this finding of blood Ach, the suggestion of using eserine for induction of labor<sup>5</sup> deserves a trial which is in progress.

**Summary.** In a study of 288 samples of blood from 152 cases, a gradual appearance of Ach toward the 2nd trimester of pregnancy, an increase of Ach with the onset of labor, and a disappearance 48 hours or more after labor were demonstrated. Blood of 20 normal subjects of both sexes showed no evidence of Ach with the same test.

<sup>2</sup> Chang, H. C., and Gaddum, J. H., *J. Physiol.*, 1933, **79**, 255.

<sup>3</sup> Meng, C. W., *Chin. J. Physiol.*, 1941, **16**, 291.

<sup>4</sup> Ettinger, G. H., Brown, A. B., and McGill, A. H., *J. Pharmacol.*, 1941, **73**, 119.

<sup>5</sup> Chang, H. C., Lee, L. Y., and Meng, C. W., *Chin. J. Physiol.*, 1940, **15**, 343.

TABLE II. Citrulline Content of and "Recovery" from Protein Digests.

Protein	% N filtrable as $\text{NH}_2\text{-N}$ after acid hydrolysis (A)	% N filtrable as $\text{NH}_2\text{-N}$ after enzyme hydrolysis (B)	% digestion (100 B/A) (C)	% Citrulline in protein (M and A free) (D)	Citrulline added to digest, mg/100 ml (E)	Citrulline found, mg/100 ml (F)	% recovery (G)
Casein	11.80	8.01	67.8	0.093	4.675* 3.260†	13.66 12.82	271 391†
Myosin	12.76	8.79	69.0	0.089	4.675* 3.260†	15.00 10.70	299 298§
Arachin	12.36	8.03	65.0	0.044	4.675* 3.260†	15.56 11.20	320 336†
Amandin	13.27	5.02	37.9	0.080	4.675* 3.260†	18.14 15.08	368 435†
Pumpkin seed globulin	12.01	8.13	67.6	0.072	4.675* 3.260†	18.66 13.51	381 385§
Watermelon seed globulin	11.75	8.12	69.1	0.066	4.675* 3.260†	17.02 11.02	346 316†

\* Present throughout digestion.

† 1 ml citrulline solution diluted to 25 ml with digest.

‡ Not boiled.  
§ Boiled.

Since these extraordinarily high "recoveries" were not obtained in the enzyme blank, substances other than the proteins or their digestion products are eliminated as a cause for the high "recovery." Amino acids cannot cause this effect since an acid hydrolysate of casein added to citrulline and put through the colorimetric determination of citrulline did not give high results. Similar but less markedly high "recoveries" were obtained when commercial peptones were added to citrulline solutions, so that it would appear that the presence of partial digestion products of the proteins in a citrulline solution markedly enhances the chromogenicity of the citrulline in the diacetyl monoxime reaction.

*Discussion.* Although the results obtained in this study are not absolutely conclusive in demonstrating that citrulline is not present in proteins, they nevertheless strongly indicate that it is absent or present only in very small amounts in the 6 proteins studied. Citrulline might be more readily destroyed by acid when in peptide combination than when in the free state, but unless this difference were very great, small amounts of it should survive acid hydrolysis. Acid hydrolysates were completely negative for citrulline by the diacetyl monoxime reaction. Furthermore, if significant amounts of citrulline were present, it should have been recovered in the enzymatic digests. The exact nature of the ureide compound present cannot be determined from these results. The virtual elimination of citrulline as a possibility enhances the probability that the original ureide may be either a cyclic type of structure or more probably simply carbamic acid present as a constituent of the peptide chain and hydrolyzed and spontaneously decomposed during enzymatic hydrolysis.

*Summary.* 1. Casein, amandin, arachin, myosin, and the crystalline globulins of pumpkin seed and watermelon seed give marked to moderately strong diacetyl monoxime reactions for citrulline when tested directly but their acid hydrolysates give completely negative results.

2. Citrulline itself is relatively slowly destroyed by treatment with 6N hydrochloric acid at 110°C.

3. Only very small amounts of ureide com-

TABLE I.  
Analysis of Protein Samples.

Protein	Moisture, %	Ash, %	N, %	N, % M and A free
Casein	10.36	0.37	13.78	15.44
Myosin	10.04	1.03	15.03	16.90
Arachin	9.44	2.86	16.01	18.26
Amandin	9.38	0.43	17.45	19.35
Pumpkin seed globulin	10.23	0.15	16.47	18.38
Watermelon seed globulin	9.31	0.06	16.77	18.50

done from a tryptic digest of casein it was decided to determine the amounts of citrulline present in enzymatic hydrolysates of the 6 proteins. Seven 1 g samples of each of the proteins were weighed out into 125 ml Erlenmeyer flasks. About 1 g of precipitated calcium carbonate was added to each flask, together with 50 ml of water. The samples were allowed to stand for 24 hours in the refrigerator in order for the protein to become thoroughly wetted. Ten mg of Difco, 1/110 trypsin were then added to each flask and to each of 7 enzyme blanks containing calcium carbonate and water. A known amount of citrulline was added to 3 of the samples of each protein and to 3 enzyme blanks; about 1 ml of xylene was added to each flask and all samples were incubated at 37°C. At intervals samples were withdrawn from one enzyme blank and from one sample (containing no added citrulline) of each protein and titrated by Northrup's<sup>10</sup> formol titration, modi-

fied in that the end points were determined with the aid of a Beckman pH meter. When the amino nitrogen contents had become constant in the pilot flasks an additional 10 mg of trypsin and 1 ml of an active Waldschmidt-Leitz<sup>11</sup> preparation of erepsin were added to each flask. Digestion and determination of the amino nitrogen in the pilot flask was continued; and when the amino nitrogen had become again constant an additional ml of erepsin was added to each flask. After one week's additional digestion the samples were removed from the incubator, filtered into 100 ml volumetric flasks, and made up to volume with the washings. Aliquots of these digests were taken for determination of total amino nitrogen content and of total ureide content. The results are shown in Table II. In each case a correction has been made for both amino nitrogen and citrulline content by subtracting the value found in the appropriate blank from that found in the protein digest. In no case was more than 0.09% of the protein accounted for as "citrulline" by the colorimetric determination; however, in all runs in which citrulline was added to the digests the recoveries in terms of color intensities were extraordinarily high, *i.e.*, the observed colorimetric values corresponded to from 300 to 500% of the amounts added. It was thought that a peptide with enhanced chromogenicity might have been formed during hydrolysis, but similarly high "recoveries" were obtained when the citrulline was added to the digests immediately before determination and even when the digests had been heated for half an hour on a boiling water bath to inactivate any residual enzyme.

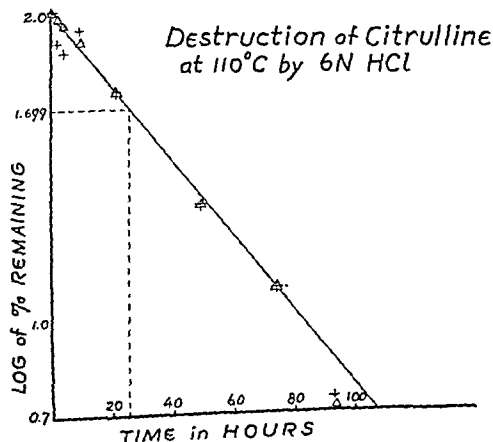


Fig. 1.

<sup>10</sup> Northrup, J. H., *J. Gen. Physiol.*, 1925-26, 9,

<sup>11</sup> Waldschmidt-Leitz, E., and Schaeffner, A., *Z. physiol. Chem.*, 1926, 151, 31.

TABLE I.  
Results of Lipid Analyses of the Arctic Stickleback and of 2 Groups of Guppies.  
All values expressed in percent fresh tissue.

Substance	Arctic stickleback	Guppies fed commercial fish food	Guppies fed earth-worms
Fatty acid—mean	4.88	8.87	7.30
Range	2.46-7.06	8.14-9.94	6.22-9.33
Cholesterol—mean	0.32	0.56	0.55
Range	0.29-0.35	0.4-0.93	0.44-0.63
Lipid P—mean	0.08	0.09	0.08
Range	0.06-0.08	0.07-0.12	0.06-0.09
Phospholipid—mean	1.87	2.31	2.05
Range	1.57-2.08	1.83-2.60	1.52-2.31

TABLE II.  
Table Showing the Various Lipid Ratios for the Arctic Stickleback and for the 2 Groups of Guppies. The ratios were calculated from the means given in previous table.

Group	Chol/fa	Chol/phosph	Chol/lipid P	Fa/lipid P
Stickleback	.07	.17	4.0	61.0
Guppy—worm fed	.08	.27	6.9	91.0
Fish food	.06	.24	6.2	99.5

3 groups is the same.

Calculations of the various lipid ratios were made for each group (Table II). Examination of these ratios indicates that, in relation to cholesterol, there is more phospholipid in the Arctic fish than in any of the guppies. It is maintained that the higher the ratio, cholesterol/lipid phosphorus, the greater the water content of the tissue in question.<sup>6</sup> If this generalization is universally true, the Arctic stickleback contains less water than does the guppy. It might be that this lower water content is correlated with the ability of the stickleback to withstand freezing.<sup>7</sup>

The fatty acid/lipid phosphorus ratios show that in the Arctic fish there is much less fatty acid, in proportion to phospholipid, than in the guppies. This may indicate that the turnover of fats is more rapid in the stickleback than in the guppies.

There is an interesting parallelism in the lipid content of the whole carcasses of guppies fed on commercial food and liver lipids in various fish. For example, Weill<sup>8</sup> found the

liver cholesterol in various fish to be as follows (percent wet weight): *Trutta fluviatilis*, 0.68; *Gadus merlangus*, 0.54; *Solea vulgaris*, 0.47. These values are quite close to that of 0.56% cholesterol in the guppies fed commercial food (Table I).

Mottram<sup>9</sup> reported that in the liver of the plaice fatty acids amount to 8.86% of the fresh tissue. This value compares closely with that of 8.87% fatty acids in whole guppies (Table I).

Similarly, Javillier and co-workers<sup>10</sup> found that in the liver of the carp lipid phosphorus amounts to 0.099% fresh tissue, a value similar to 0.09% for lipid phosphorus in the whole guppies. The Arctic stickleback also contains a similar amount of lipid phosphorus 0.08% (Table I), although its other lipids do not resemble so closely those of the guppy.

The present results indicate that in the stickleback phospholipids are high in proportion to other lipids. Such a condition suggests that in the single Arctic fish studied lipid metabolism is proceeding at a higher level than in the guppies, for phospholipid level is

<sup>6</sup> Mayer, A., and Schaeffer, G., *J. physiol. path. gen.*, 1913, 15, 771.

<sup>7</sup> Pouchet, F. A., *J. Anat. Physiol.*, 1866, 3, 1.

<sup>8</sup> Weill, J., *C. R. Acad. Sci.*, 1914, 158, 642.

<sup>9</sup> Mottram, V. H., *J. Physiol.*, 1912, 45, 363.

<sup>10</sup> Javillier, M., Cremieu, A., and Hinglais, A., *Bull. Soc. chim. Biol.*, 1925, 10, 327.



pounds are found in enzymatic hydrolysates of the 6 proteins; however, when citrulline is added to such hydrolysates, its "recovery" in terms of colorimetric units corresponds to several times the theoretical value. The reason for this anomalous result has yet to be determined.

4. It is probable that citrulline is either

absent or present only in minimal quantities in these proteins.

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The authors wish to thank Miss Jane Price for the nitrogen analyses of the protein preparations.

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## Comparative Study of Lipids in Whole Carcasses of Arctic and Non-Arctic Fish.\* (17453)

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Information concerning lipid content and metabolism in cold-blooded animals is relatively meager.<sup>1</sup> Concerning these matters in Arctic poikilotherms, information is non-existent. A survey was, therefore, made of the lipid content of whole fish, *Pygosteus pungitius*,<sup>2</sup> the Arctic 9-spined stickleback. The results were evaluated in comparison with similar analyses made on the common guppy (*Girardinus guppyi*).

**Material and methods.** Whole sticklebacks or guppies were carefully weighed and then dropped into a mixture consisting of 3 parts of pure ethanol and 1 part benzene. After 24 hours the fish were ground with sand and the lipids extracted according to Bloor's method.<sup>1</sup> Total fatty acids were estimated using the method of Bloor as modified by Snell.<sup>3</sup> Lipid phosphorus was estimated by a modified Youngburg technic,<sup>4</sup> from the latter results

phospholipids were calculated as lecithin. Measurements of cholesterol were based on methods developed by Bloor.<sup>5</sup> Since all the procedures are colorimetric, a Klett-Summers photometer was used to measure the various color intensities. Ten sticklebacks were analyzed. Two groups of 5 each guppies were tested: one group was fed ordinary commercial fish food; the other, chopped earthworms.

**Results.** The results are summarized in Table I, which shows that there is a mean value of 4.88% fatty acids, 0.32% cholesterol, and 1.87% phospholipids in the Arctic stickleback.

Table I also shows that the group of guppies fed on commercial fish food have the following mean lipid values: fatty acids, 8.87%; cholesterol, 0.56%; phospholipid, 2.31%. Guppies fed on chopped worms have lipid values practically identical with those fed commercial fish food.

**Discussion.** The results indicate that there is about the same amount of cholesterol in the guppies on either diet, but that there is less cholesterol in the Arctic fish than in the guppies. Total fatty acids are higher in the guppies, on either diet, than in the Arctic stickleback. The phospholipid content of all

\* Supported by a grant from the Arctic Institute of North America with funds provided by the Office of Naval Research. Sincere thanks are due Dr. Laurence Irving, director, and the staff of the Arctic Research Laboratory for invaluable aid rendered during the work in the field.

<sup>1</sup> Bloor, W. R., *Biochemistry of the Fatty Acids*, New York, 1943.

<sup>2</sup> Preble, E. A., A biological investigation of the Athabaska-Mackenzie Region, North American Fauna, No. 27, Washington, 1908.

<sup>3</sup> Snell, F. D., and Snell, C. T., *Colorimetric methods of analysis*, New York, 1937.

<sup>4</sup> Youngburg, G. E., and Youngburg, M. V., *J. Lab. Clin. Med.*, 1930, 16, 158.

<sup>5</sup> Bloor, W. R., *J. Biol. Chem.*, 1916, 24, 227.

nitude of change.

Bipolar registration, shifting of electrodes, use of needle electrodes or coupling of 2 to 8 electrodes did not yield any essential improvement. The changes in the records from the base of the skull were similar to those observed from the cortical area.

In 4 experiments with morphine an additional decrease occurred to some extent on the application of the pain stimulus. Further tests were not made because pain and morphine<sup>2</sup> tend to produce both flattening of the middle range of waves which makes reliable

<sup>2</sup> Gibbs, F. A., and Maltby, G. L., *J. Pharm. and Exp. Therap.*, 1943, **78**, 1.

interpretation of experimental data practically impossible.

*Summary.* The possibility of E.E.G. registration of pain was examined in 17 human subjects. A decrease in amplitude of the waves was observed, which was more evident in the parietal, occipital, temporal and frontal in a descending order. This change, however, is not dependent on the nature and degree of pain and it is not specific as it occurred with cold, heat and touch as well.

We desire to acknowledge the assistance of Dr. F. A. Gibbs and Mrs. E. L. Gibbs in certain aspects of this study.

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## Experimental Pulmonary Edema. II. Pathogenesis of Pulmonary Edema Caused by Ammonium Ion.\* (17455)

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Koenig and Koenig<sup>1</sup> have described an acute pulmonary edema following the administration of ammonium salts to different species. It appears to be specifically a result of the ammonium ion. The occurrence and gross appearance of this edema resembles epinephrine induced pulmonary edema and in an effort to obtain information about the mechanism of the ammonium pathology a study has been made of its relation to edema due to epinephrine and adrenergic stimuli.

*Experimental.* Epinephrine induced pulmonary edema may be prevented by adrenergic blocking agents.<sup>2,3</sup> Their influence was observed upon ammonium pulmonary edema. Unfasted guinea pigs were divided into groups with the same sex distribution. All groups

were lightly etherized to pass a stomach tube. Group 1, the controls, were given a sham dose of 10 cc water per kg body weight. All other groups received 1200 mg/kg of a 12% solution of  $\text{NH}_4\text{Cl}$  by oral gavage. Ten minutes before receiving the ammonium salt groups 3, 4 and 5 respectively received by intravenous injection in 1 cc saline/kg; 2 mg/kg of a B-haloethylamine, N-(9-fluorenyl)-N-ethyl-B-chloroethylamine-HCl ("SKF-501"); 2.5 mg/kg N-(2-chloroethyl)-N-ethylbenzhydramine-HCl ("SY-2"), which like "SKF-501" is an effective adrenergic blocking agent; and 1.5 mg/kg of the potent antihistaminic N-B-dimethylaminopropylthiodiphenylamine - HCl ("Phenergan", 3277 R.P.). The presence of pulmonary edema was determined at an autopsy by gross inspection and lung weight was used as a measure of its extent.

The summarized data comprise Table I. Group 1 was killed with ether while all of the animals in the other groups succumbed with convulsions 5-30 minutes after receiving the ammonium salt. The two adrenergic blocking agents and the antihistaminic all were

\* This study was supported by a grant from the Life Insurance Medical Research Fund.

<sup>1</sup> Koenig, H., and Koenig, R., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 375.

<sup>2</sup> Stone, C. A., and Loew, E. R., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 122.

<sup>3</sup> MacKay, E. M., and Pecka, E. F., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 669.

a good indication of overall level of fat metabolism.<sup>1</sup> One might expect just such a condition as an adaptation on the part of the stickleback to Arctic living: the high caloric values of the fats make them ideal sources of energy in an environment such as the Arctic.

Other adaptations of a metabolic nature are known to exist in Arctic fish: polar cod tissue shows  $Q_{O_2}$ -temperature curves peculiarly adapted to functioning at low temperatures.<sup>11</sup>

*Summary.* 1. Whole Arctic sticklebacks and ordinary guppies were analyzed for fatty acids, cholesterol and lipid phosphorus.

2. It was found that diet does not have an appreciable effect on the lipids in the guppies.

3. Cholesterol and fatty acid are higher in the guppies than in the Arctic fish. On the other hand phospholipid is essentially the same in all the fish examined.

4. There is more phospholipid in relation to cholesterol in the Arctic sticklebacks than in the guppies, a fact which may be interpreted as indicating a higher level of fat turnover in the former than in the latter.

5. The ratio of cholesterol/lipid phosphorus is lower in the stickleback than in the guppy, indicating a lower water content in the tissues of the former. This may be correlated with the reported ability of the stickleback to resist freezing injuries.

<sup>11</sup> Field, J., and Peiss, C. N., *Fed. Proc.*, 1949, 8, 44.

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## Electroencephalographic Changes Associated with Painful and Non-Painful Peripheral Stimulation. (17454)

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This work was undertaken to ascertain whether the perception of pain is associated with a specific change in the electroencephalogram (E.E.G.), which might be used as an objective criterion of pain.

*Methods.* A Grass Electroencephalograph was used with 6 or 8 electrodes in 17 experiments on as many subjects. In addition to the usual 8 electrodes (2 frontals, 2 parietals, 2 temporals and 2 occipitals) the Grinker needle electrode<sup>1</sup> in the sphenoid bone was employed in 2 experiments, and a saline saturated cotton electrode pressed against the posterior pharyngeal wall in one. For the purpose of exploring any changes from the region of the base of the skull or hypothalamus near the foramen ovale in still another experiment an insulated needle electrode was inserted (under infiltration anesthesia) between the mandible and zygoma to a depth of 5.5 cm. Monopolar registration was employed with the lobe of the ear as indifferent electrode. Pain was produced in several ways in each experiment. (a) A Von Frey hair stimulator

was applied to a previously determined pain point on the right forearm with a pressure of 8 g. (b) The web of the skin between the thumb and index finger of the right hand was pressed between a forceps. (c) As a control in two experiments heat, cold and touch were applied to the right forearm without causing pain.

*Results.* In all experiments a definite change in the E.E.G. occurred. It consisted of a decrease in amplitude, which was in the nature of a loss of electrical entropy and must be separated from the attention wave, which frequently appeared as an additional factor. The change was not specific for a pain stimulus or sensation since a similar response was obtained with heat, cold and touch. The decrease was bilateral and was not confined to any large cortical area, though it was more pronounced in the parietal, occipital and temporal region in a decreasing order of mag-

<sup>1</sup> Grinker, R. R., and Serota, H. M., *J. Neurophysiol.*, 1938, 1, 573.

TABLE II.  
Influence of Ammonium Chloride on Blood Sugar.

Time in min.	1 Controls	2 NH <sub>4</sub> Cl	Average blood sugar concentration—mg%*	
			3† NH <sub>4</sub> Cl preceded by "SKF-501"	4‡ NH <sub>4</sub> Cl preceded by tetraethylammonium
0	112	132	140	136
10	116	216	224	206
17	118		208	
20			260	220
22		272	256	244
24	120	296	272	282

\* 3 unfasted guinea pigs in each group.

† Doses of NH<sub>4</sub>Cl and "SKF-501" as in Table I.

‡ Dose of tetraethylammonium bromide 10 mg/kg subcutaneously.

monium chloride and it was prevented by the adrenergic blocking agents. The control lungs always appeared much more edematous than their weight would indicate. We have often noted a disproportion between the extent of edema and the amount of fluid which collected in the alveoli and bronchioles. Groups of four 2 kg rabbits were given 1.25 g/kg of 25% NH<sub>4</sub>Cl by stomach tube. Two groups were given the blocking agents in the same dose used in the guinea pig experiments. The lungs of Group 1 (controls) averaged 0.46 g, Group 2 (NH<sub>4</sub>Cl treated) 0.71 g, Group 3 (NH<sub>4</sub>Cl preceded by "SKF-501", see Group 3, Table I) 0.45 g and Group 4 (NH<sub>4</sub>Cl preceded by "SY-2", see Group 4, Table I) 0.42 g/100 g body weight, respectively. There was no edema except in the lungs of Group 2.

Ammonium salts might exert an adrenergic effect by direct action on sympathetic nerve endings, or by direct or reflex central stimulation. Ammonia has long been known as a most effective means of stimulating the brain medulla reflexly<sup>6</sup> and in addition to lung edema, the Koenigs have found pathology resulting from fatal doses of ammonium salts. Hypnotics have been claimed to prevent lung edema due to epinephrine.<sup>7</sup> Ethyl ether produces an anesthesia characterized by complete paralysis of the motor reflex centers in the cord and some depression of the medullary centers. When our animals were continued under a deep ether anesthesia in place of stopping the

anesthetic after the light etherization used for gavage to give the ammonium chloride, they lived as long as similar unanesthetized controls and died without the usual convulsions typical of toxic doses of ammonium salts. Pulmonary edema was always absent. The average lung weight of 4 etherized ammonium chloride pigs was 0.57 in comparison with 1.34 g/100 g body weight for a similar group of non-etherized NH<sub>4</sub>Cl controls. We interpret this as evidence in favor of the ammonium-induced pulmonary edema being a result of reflex or direct stimulation of the medullary centers which results in an outflow of excessive adrenergic stimuli. The stimuli do not necessarily act on the pulmonary circulation primarily but as in epinephrine pulmonary edema<sup>8</sup> may produce left heart failure with an increased pulmonary venous pressure and the inevitable edema.

The administration of ammonium chloride causes a marked hyperglycemia which might be interpreted as due to a release of epinephrine by the adrenal medulla. However ammonium salts have been reported<sup>9,10</sup> to have no effect on adrenal release of epinephrine and they may induce a hyperglycemia in adrenalectomized rabbits.<sup>10</sup> We have found (Table II) that neither an adrenergic blocking agent nor tetraethylammonium which blocks the release of epinephrine by the adrenal medulla<sup>11</sup> influences the hyperglycemia due to ammoni-

<sup>6</sup> Sollman, T., *A Manual of Pharmacology*, 3rd ed., p. 559, W. B. Saunders Co., Philadelphia, 1928.

<sup>7</sup> Luisada, A., *Arch. Exp. Path.*, 1928, **132**, 313.

<sup>8</sup> Houssay, B. A., and Molinelli, E. A., *Compt. rend. Soc. biol.*, 1925, **93**, 1156.

<sup>9</sup> Shoji, J., *Tohoku J. Exp. Med.*, 1937, **30**, 259.

<sup>10</sup> Satake, Y., *Tohoku J. Exp. Med.*, 1926, **8**, 26.

TABLE I.  
Prevention of Ammonium Chloride Pulmonary Edema in Guinea Pigs with Adrenergic Blocking Agents.

Group	No. of animals		Avg body wt, g	Lung wt, g/100 g body wt
	In group	With edema		
1	18	0	736	0.69 $\pm$ .11
2	24	24	1200 mg NH <sub>4</sub> Cl/kg 680	1.50 $\pm$ .21
3	18	0	1200 mg NH <sub>4</sub> Cl/kg preceded by 2 mg/kg of N-(9-fluorenyl)-N-ethyl-B-chlorethylamine*	0.71 $\pm$ .11
4	12	0	1200 mg NH <sub>4</sub> Cl/kg preceded by 2.5 mg/kg N-(2-chloroethyl)-N-ethylbenzhydrylamine · HCl†	0.65 $\pm$ .15
5	12	11	1200 mg NH <sub>4</sub> Cl/kg preceded by 1.5 mg/kg N-B-dimethylaminopropylthiodiphenylamine · HCl‡	1.40 $\pm$ .25

\* We are indebted to Dr. Glenn E. Ulliot, of Smith, Kline and French in Philadelphia for a supply of this compound (SKF-501).

† Dr. Leon A. Sweet of Parke, Davis and Co., Detroit, Mich., sent this material (SY-2) to us.

‡ (Phenergan, 3277 R.P.) supplied through the courtesy of Dr. A. Gibson, Assoc. Medical Director, Merck and Co., Rahway, N. J.

without influence upon mortality. Since both of the adrenergic blocking agents completely prevented the ammonium edema but not the mortality, we may conclude that pulmonary edema need not be the cause of death from toxic doses of ammonium salts. The antihistaminic was without effect on the ammonium edema, which in this respect resembles the fulminating pulmonary edema due to epinephrine.<sup>2</sup>

Epinephrine-induced pulmonary edema appears to be due to increased pulmonary pressure which it produces<sup>2</sup> possibly not directly, even though the vascular tree of the lungs is richly supplied with sympathetic fibres, but as a result of the high systemic arterial pressures. The complete protection against epinephrine edema afforded by adrenergic blocking drugs<sup>2,3</sup> which pharmacologically block excitatory actions of epinephrine such as vasoconstriction, is also true of the ammonium edema which suggests that the latter might also result from adrenergic stimuli. Some years ago we suggested<sup>4</sup> that the pulmonary edema of experimental hypoglycemia might be due to the release of epinephrine by the adrenal medulla. If this were true, medullectomy of the

adrenal in the rat, where this procedure is feasible, could affect the development of the ammonium edema. We tried to test this but were unable to produce this type of pulmonary edema in our rats (Slonaker Strain<sup>5</sup>). Groups of 6 rats each had lung weights of 0.53  $\pm$  .06 g/100 g body weight (controls), 0.55  $\pm$  .07 g/100 g body weight (normals receiving 0.4 cc 10% NH<sub>4</sub>Cl/100 g body weight intraperitoneally) and 0.48  $\pm$  .09 g/100 g body weight (demedullectomized rats receiving the same dose of NH<sub>4</sub>Cl), respectively. This was equivalent to the dose of ammonium salt used by the Koenigs to produce edema in their rats. Nor could we produce pulmonary edema in the rat with ammonium salts whatever the dose when given *per os* as well as intraperitoneally. This is additional evidence that mortality from ammonium salts is not necessarily a result of the pulmonary edema which they frequently cause, since our rats all died. The same thing was true in albino mice of an unknown strain. We also are in disagreement with the Koenigs as to the action of ammonium salts in the rabbit. They found no edema. We regularly found lung edema after the administration of am-

<sup>5</sup> MacKay, L. L., and MacKay, E. M., *Am. J. Physiol.*, 1927, **83**, 179.

<sup>4</sup> Sherrill, J. W., and MacKay, E. M., *Arch. Int. Med.*, 1939, **64**, 907.

serum globulins in normal mice exposed to a single dose of X-rays.<sup>3</sup> It was suggested that the influence of radiation on blood globulin levels in intact mice was chiefly a result of the direct involution of lymphoid tissue by large radiation doses. An augmentation of pituitary-adrenal cortical controlled lymphocytolysis, with accompanying blood globulin elevation, was also demonstrated to occur as a result of the response of the endocrine mechanism to the action of radiation as a non-specific stimulus.

A review of the literature<sup>4</sup> indicates that conflicting data have been obtained in efforts to establish definitely a relationship between pituitary-adrenal cortical secretion and serum protein levels. Simonnet<sup>5</sup> injected adrenotrophic hormone or adrenal cortical extracts into rats of both sexes and of varying ages, and found a rise in total blood globulin occurring most consistently in the 2-month-old male rat. On the other hand, Li and Reinhardt<sup>6</sup> were unable to alter the electrophoretic serum protein pattern of normal or of hypophysectomized rats by giving single or chronic injections of adrenotrophic hormone. It may be noted that the data of these investigators show an approximately 100% difference in values for  $\gamma$ -globulin concentrations in 2 groups of control rats.

In rats and dogs exposed to physical and chemical stimuli which augment pituitary-adrenal cortical secretion, Chanutin and his associates<sup>7,8</sup> found rather uniformly an increase in the serum  $\alpha$ -globulin fraction and the appearance of boundary anomalies in the  $\beta$ -globulin component. Arocha and De Venanzi<sup>9</sup> reported briefly that serum globulin increased

in normal dogs following intravenous injections of epinephrine, and Mondolfo and DeLerner<sup>10</sup> confirmed the observations of Chase, White, and Dougherty<sup>11</sup> that adrenal cortical extract injections, accompanying antigen administration, resulted in a greater rate and degree of elevation of blood immune globulin. However, Eisen and co-workers<sup>12</sup> found identical concentrations of serum antibodies and  $\gamma$ -globulin in adrenalectomized rats injected repeatedly during immunization with adrenal cortical extract, and in operated animals not receiving hormone.

Pituitary adrenotrophic hormone or adrenal cortical extract injections have failed generally to influence the level of serum globulin in human subjects. This has been the experience of Kelley and Adams,<sup>13</sup> Forsham and co-workers,<sup>14</sup> Mason and co-workers<sup>15</sup> and Frieden and White,<sup>16</sup> following administration of adrenotrophic hormone or adrenal cortical extracts. In addition, exposure of patients to X-radiation, nitrogen mustard therapy, or electric shock treatment was rather uniformly without influence on the electrophoretic pattern of the serum proteins, although in two instances significant rises were seen in the serum  $\gamma$ -globulin fraction after x-radiation.<sup>16</sup>

In view of the nature of the conflicting evidence in a variety of species, experiments have been initiated to investigate systematically the possible variables which may be involved in influencing the nature and extent of the response of the serum protein pattern to circumstances of augmented levels of cir-

<sup>3</sup> Dougherty, T. F., and White, A., *Endocrinology*, 1946, **39**, 370.

<sup>4</sup> White, A., *Ann. Rev. Physiol.*, 1949, **11**, 355.

<sup>5</sup> Simonnet, H., *Bull. Acad. Med. (Paris)*, 1947, **131**, 245.

<sup>6</sup> Li, C. H., and Reinhardt, W. O., *J. Biol. Chem.*, 1947, **167**, 487.

<sup>7</sup> Chanutin, A., and Gjessing, E. C., *J. Biol. Chem.*, 1946, **165**, 421.

<sup>8</sup> Gjessing, E. C., Ludewig, S., and Chanutin, A., *J. Biol. Chem.*, 1947, **170**, 551.

<sup>9</sup> Arocha, H. G., and De Venanzi, F., *Proc. Intern. Physiol. Congr.*, 1949, XVII Congress, 129.

<sup>10</sup> Mondolfo, H., and De Lerner, S. J., *Farmacologia*, 1947, **2**, 171.

<sup>11</sup> Chase, J. H., White, A., and Dougherty, T. F., *J. Immunol.*, 1946, **52**, 101.

<sup>12</sup> Eisen, H. N., Mayer, M. M., Moore, D. H., Tarr, R. R., and Stoerk, H. C., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 301.

<sup>13</sup> Kelley, V. C., and Adams, J. M., *J. Pediat.*, 1948, **32**, 282.

<sup>14</sup> Forsham, P. H., Thorn, G. W., Prunty, F. T. G., and Hills, A. G., *J. Clin. Endocrinology*, 1948, **8**, 15.

<sup>15</sup> Mason, H. L., Power, M. H., Ryncarson, E. H., Ciaramelli, L. C., Li, C. H., and Evans, H. M., *J. Clin. Endocrinology*, 1948, **8**, 1.

<sup>16</sup> Frieden, J., and White, A., unpublished results.

um salts in guinea pigs and conclude that the carbohydrate disturbance is not directly related to the mechanism of the pulmonary edema.

**Summary 1.** The acute pulmonary edema which follows the administration of ammonium salts to guinea pigs may be entirely prevented by prior administration of the adrenergic blocking agents, N-(9-fluorenyl)-N-ethyl-B-chloroethylamine-HCl ("SKF-501") and N-(2-chloroethyl)-N-ethyl-benzhydramine-HCl ("SY-2"). Less marked lung edema is produced in rabbits by ammonium chloride and it is also prevented by the blocking agents.

2. A potent antihistaminic agent, N-B-dimethylamino-propylthiodiphenylamine-HCl ("Phenergan-3277 R.P."), was without effect on the incidence of ammonium pulmonary edema, which in this respect as well as the manner it is influenced by adrenergic blocking agents, resembles epinephrine-induced pulmonary edema.

3. A central nervous system depressant in the form of continuous ether anesthesia prevents the occurrence of ammonium pulmonary edema. Along with the effect of adrenergic blocking agents this is construed as evidence

for the mechanism of this edema being due to adrenergic stimuli of reflex or direct central stimulation origin.

4. Ammonium salts cause a striking hyperglycemia. Since adrenergic blocking agents of the type used as well as tetraethylammonium which blocks epinephrine release by the adrenal medulla fail to prevent the hyperglycemia response it is believed that ammonium hyperglycemia is not directly related to the edema formation.

5. Although pulmonary edema sometimes may be the cause of death from toxic doses of ammonium salts, this is not true in the Slonaker strain of rats or our strain of albino mice both of which fail to develop edema. Nor is this the cause of death in guinea pigs or rabbits when an adrenergic blocking agent is used to prevent the edema, as the animals succumb as a result of other effects of the ammonium ion.

**Addendum:** When this manuscript was ready for submission to the Editors, an article by the Koenigs (Koenig, H., and Koenig, R., *Am. J. Physiol.*, 1949, **158**, 1) on the pathogenesis of ammonium pulmonary edema appeared. Insofar as our observations are similar the conclusions are parallel.

<sup>11</sup> Morrison, J. L., and Farrar, C. H., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 235.

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## Effect of Adrenal Cortical Extract and of X-Radiation on Serum Proteins of Mice. (17456)

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It has been reported previously from this laboratory that chronic injections of pituitary adrenotrophic hormone in mice produced an

increase in the total serum proteins.<sup>1</sup> Moreover, a single injection of adrenotrophin or of adrenal cortical steroids in oil in rabbits was observed to cause an elevation of circulating  $\beta$ - and  $\gamma$ -globulins within a few hours after hormone administration.<sup>2</sup> Electrophoretic analysis also revealed an increase in these

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<sup>1</sup> White, A., and Dougherty, T. F., *Proc. Soc. Exp. Biol. and Med.*, 1944, **56**, 26.

<sup>2</sup> White, A., and Dougherty, T. F., *Endocrinology*, 1945, **33**, 207.

serum globulins in normal mice exposed to a single dose of X-rays.<sup>3</sup> It was suggested that the influence of radiation on blood globulin levels in intact mice was chiefly a result of the direct involution of lymphoid tissue by large radiation doses. An augmentation of pituitary-adrenal cortical controlled lymphocytolysis, with accompanying blood globulin elevation, was also demonstrated to occur as a result of the response of the endocrine mechanism to the action of radiation as a non-specific stimulus.

A review of the literature<sup>4</sup> indicates that conflicting data have been obtained in efforts to establish definitely a relationship between pituitary-adrenal cortical secretion and serum protein levels. Simonnet<sup>5</sup> injected adrenotrophic hormone or adrenal cortical extracts into rats of both sexes and of varying ages, and found a rise in total blood globulin occurring most consistently in the 2-month-old male rat. On the other hand, Li and Reinhardt<sup>6</sup> were unable to alter the electrophoretic serum protein pattern of normal or of hypophysectomized rats by giving single or chronic injections of adrenotrophic hormone. It may be noted that the data of these investigators show an approximately 100% difference in values for  $\gamma$ -globulin concentrations in 2 groups of control rats.

In rats and dogs exposed to physical and chemical stimuli which augment pituitary-adrenal cortical secretion. Chanutin and his associates<sup>7,8</sup> found rather uniformly an increase in the serum  $\alpha$ -globulin fraction and the appearance of boundary anomalies in the  $\beta$ -globulin component. Arocha and De Venanzi<sup>9</sup> reported briefly that serum globulin increased

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<sup>10</sup> Mondolfo, H., and De Lerner, S. J., *Farmalecta*, 1947, **2**, 171.

<sup>11</sup> Chase, J. H., White, A., and Dougherty, T. F., *J. Immunol.*, 1946, **52**, 101.

<sup>12</sup> Eisen, H. N., Mayer, M. M., Moore, D. H., Tarr, R. R., and Stoerk, H. C., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 301.

<sup>13</sup> Kelley, V. C., and Adams, J. M., *J. Pediat.*, 1948, **32**, 282.

<sup>14</sup> Forsham, P. H., Thorn, G. W., Prunty, F. T. G., and Hills, A. G., *J. Clin. Endocrinology*, 1948, **8**, 15.

<sup>15</sup> Mason, H. L., Power, M. H., Ryncarson, E. H., Ciaramelli, L. C., Li, C. H., and Evans, H. M., *J. Clin. Endocrinology*, 1948, **8**, 1.

<sup>16</sup> Frieden, J., and White, A., unpublished results.

<sup>3</sup> Dougherty, T. F., and White, A., *Endocrinology*, 1946, **30**, 370.

<sup>4</sup> White, A., *Ann. Rev. Physiol.*, 1949, **11**, 355.

<sup>5</sup> Simonnet, H., *Bull. Acad. Med. (Paris)*, 1947, **131**, 245.

<sup>6</sup> Li, C. H., and Reinhardt, W. O., *J. Biol. Chem.*, 1947, **167**, 487.

<sup>7</sup> Chanutin, A., and Gjessing, E. C., *J. Biol. Chem.*, 1946, **165**, 421.

<sup>8</sup> Gjessing, E. C., Ludewig, S., and Chanutin, A., *J. Biol. Chem.*, 1947, **170**, 551.

<sup>9</sup> Arocha, H. G., and De Venanzi, F., *Proc. Intern. Physiol. Congr.*, 1949, XVII Congress, 129.



culating adrenal cortical steroid hormones. The present investigation deals with the response in mice. The previously reported<sup>8</sup> electrophoretic experiments with serum obtained from mice exposed to X-radiation were conducted in phosphate buffer at pH 7.8. Under these circumstances, the separation of the  $\gamma$ -globulin component from the salt boundary is unsatisfactory, and errors may arise in evaluating correctly the contribution of the  $\gamma$ -globulin component to the total area in this region of the electrophoretic pattern. In addition, when this phosphate buffer is employed, a satisfactory separation of the  $\alpha$ -globulin fraction into its two commonly recognized individual components is not obtained. Another possible source of error in electrophoretic studies of sera results from the fact that the precise concentration of the  $\beta$ -globulin component may be somewhat obscured by the presence of lipid in this protein fraction, resulting in an apparent, but not true, increase in  $\beta$ -globulin concentration. This factor would seem to be of particular significance in circumstances of augmented pituitary-adrenal cortical secretion, particularly since the adrenal cortical steroids produce a mobilization of lipid to the liver.

The present study has involved the electrophoretic examination of the serum proteins of normal mice, and of mice bearing a transplantable lymphosarcoma, both prior to and following either the injection of adrenal cortical extract, or exposure to a single, relatively large dose of X-radiation. Under the experimental conditions employed, no significant alterations were seen in the serum protein patterns as a consequence of exposure of the animals to either the hormonal or radiation treatment.

**Methods.** Mice of both sexes of the CBA (Strong) strain, 8 to 13 weeks of age, were used in all experiments. The animals were maintained on a diet of Purina Fox Chow supplemented with calf meal; food and water were available at all times. The tumor employed was obtained from Professor W. U. Gardner of the Department of Anatomy at Yale University. This tumor arose in an estrogen-treated mouse of the C<sub>3</sub>H strain and has been transplanted for many successive

generations. A transplant of the tumor takes in 100% of CBA mice. The transplanted tumor grows rapidly and kills the host in approximately 3 weeks. The tumor has been described in detail<sup>17</sup> and is composed of large cells which resemble reticular cells. Tumor transplants were made by implanting small pieces of the tumor subcutaneously. Since the mice with this tumor transplant live generally a maximum of three weeks, the tumor-bearing animals were used 2 weeks after the transplant had been made, at a time when a clearly palpable tumor was present.

Two preparations of adrenal cortical extract were used, one an aqueous adrenal cortical extract (Wilson) and the other a preparation of adrenal cortical steroids in oil (Upjohn). The animals subjected to X-rays received total body radiation. This was accomplished by placing 4 to 6 mice in a light cardboard box, which because of its shallowness prevented the mice from shielding one another. Radiation of all animals was delivered at the rate of 43 r per minute by using the following conditions: 125 Kv., 5 ma.; 30 cm skin target distance; 3 mm aluminum filter.<sup>†</sup>

In order to obtain blood samples, each mouse was pithed, and the heart exposed and blood obtained by direct heart puncture, using a finely drawn glass pipette. Blood was pooled from groups of animals varying in number from 15 to 32, in order to obtain adequate serum for each electrophoretic experiment. The total serum protein and non-protein nitrogen contents of the pooled serum were determined by the micro-Kjeldahl technic. The serum was then diluted with 3 parts of 0.1 M sodium barbital buffer, pH 8.6, and dialyzed against the buffer for 2 or 3 days in the ice box. Electrophoretic runs were conducted in the Tiselius apparatus at 2°C for a period of approximately 3 to 4 hours, at which time satisfactory separation of the individual serum protein components had occurred. The concentrations of the various protein fractions were estimated from the descending diagrams.

<sup>17</sup> Gardner, W. U., Dougherty, T. F., and Williams, W. L., *Cancer Research*, 1944, 4, 73.

<sup>†</sup> Grateful acknowledgement is made to Dr. H. S. Kaplan for assistance in radiation of the animals.

TABLE I.  
Electrophoretic Analyses of Mouse Serum.

Electrophoretic analyses of mouse sera.															
Electrophoresis exp. No.	Animal group type	No. of mice for pooled sera	Total protein, g/%	Albumin, % g	Globulins										
					$\alpha_1$		$\alpha_2$		$\beta$		$\gamma$		$\beta + \gamma$		
					%	R	%	R	%	R	%	R	%	R	
1	Normal, ♂	32	4.53	59.70	2.70	15.70	.71	9.45	.43	9.45	.43	3.48	.16	12.95	.59
2	"	23	4.82	63.43	3.05	16.50	.79	6.74	.32	9.75	.47	3.61	.17	13.76	.64
3	Normal, ♀	15	4.75	67.60	3.21	11.63	.55	6.71	.32	10.62	.51	3.35	.16	13.97	.67
4	Tumor, ♂	15	3.57	61.36	2.19	18.80	.67	5.45	.19	10.90	.39	2.48	.09	13.38	.48
5	"	15	3.41	63.44	2.16	14.75	.50	7.05	.24	11.53	.39	3.21	.11	14.74	.50
6	Normal, ♂ and ♀ <sup>1</sup>	16	6.08	63.05	3.84	18.22	1.11	5.61	.34	10.75	.65	2.39	.15	13.14	.80
7	Tumor, ♂ + ♀ <sup>2</sup>	16	5.18	61.98	3.22	16.81	.87	5.43	.28	13.05	.68	2.73	.14	15.78	.82
8	"	18	5.22	65.80	3.43	15.42	.81	3.72	.19	11.54	.60	3.72	.19	15.26	.79
9	Normal, ♂ + ♀ <sup>3</sup>	18	4.96	66.20	3.28	15.10	.75	4.04	.20	11.60	.57	3.03	.15	14.63	.72
10	"	18	5.86	68.40	4.01	12.55	.73	5.20	.31	10.82	.63	3.03	.18	13.85	.81
16 hr after 0.2 cc adrenal cortical steroids (oil), subcut.															
23 hr after 0.5 cc " " "															
23 hr after 1.0 cc aqueous adrenal cortical extr., subcut.															
16 hr after X-irradiation (200 r).															

1 6 hr after 0.2 cc adrenal cortical steroids (oil), subcut.  
 2 3 hr after 0.5 cc " " "  
 3 3 hr after 1.0 cc aqueous adrenal cortical extr., subcut.  
 4 6 hr after X-radiation (200 r).

of the electrophoretic patterns.

**Results and discussion.** Table I presents the results of the various analyses under the experimental conditions indicated in the table. It will be seen from the data that a single injection of adrenal cortical extract, or the exposure of mice to 200 r produced an increase in the total serum protein concentration, as compared to appropriate controls, in all experiments except that designated as No. 9. This increase is in agreement with previously published data.<sup>1</sup> However, it will also be seen from the table that no significant alteration occurred in the percentage distribution of the various serum protein components as determined electrophoretically. The slight alterations which were evident in several of the experiments, *e.g.*, No. 7, occurred in the  $\beta$ -globulin fraction. In view of the influence of altered lipid concentrations on the value for this protein component, it is unlikely that this increase is indicative of an elevated concentration of  $\beta$ -globulin.

It is unknown at the present time whether the relatively low initial level of total serum proteins in the control animals was a factor in determining possible responsiveness to the experimental conditions employed. In all experimental groups, there was a striking elevation in total serum proteins. It is possible that in a relatively protein-depleted animal, there is a generalized stimulus to protein production following hormone administration or exposure to X-radiation. However, in the experimental animals, there was no significant alteration in the relative distribution of the individual serum components.

The precise relation of pituitary-adrenal cortical secretion to the normal pattern of serum proteins would appear to wait further investigation. The conflicting data in the literature, taken together with the present experiments, suggest that variables exist which remain for further careful evaluation. These comments relate not only to the pattern of normal serum globulins, but also to immune globulins, about which there also have appeared conflicting data.

It may be pointed out that serum protein levels alone do not provide a complete evaluation of the possible relation of endocrine secre-

tions to blood protein production and utilization. Thus, it has been demonstrated recently<sup>18</sup> that although the antibody level in the blood of immunized, normal and adrenalectomized rats may be at similar levels, the tissues of the adrenalectomized rats are producing antibody at a rate which is distinctly less than that of the unoperated animals. This suggests that the adrenalectomized animals are also removing antibody from the circulation at a rate which is less than normal. Thus, although the blood titer is similar in the two groups of animals, the presence of the adrenal does appear to influence the rate at which the antibody globulin enters and leaves the circulation. It may also be of significance that Mondolfo and DeLerner<sup>10</sup> reported recently

<sup>18</sup> Roberts, S., and White, A., unpublished results.

that whereas relatively small doses of adrenal cortical extract may augment the rate of antibody production, significantly larger amounts of the same extract produced a suppression of antibody production as compared to the rate seen in animals receiving antigen alone.

*Summary.* Normal mice, or mice bearing a transplantable lymphosarcoma, were given single injections of adrenal cortical extract or exposed to a single radiation dose of 200 r (total body radiation). Examination of the serum protein patterns of these mice at 3 and at 6 hours after hormone injection, or at 6 hours after exposure to X-rays, revealed a striking increase in total serum protein levels. However, electrophoretic examination did not reveal significant alterations in the relative distribution of the serum proteins.

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## Cinchophen Cholerisis and Production of Peptic Ulcer in Various Species. (17457)

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Brugsch and Horsters<sup>1</sup> were the first to show that cinchophen, in both dogs and man caused an increase in the bile flow. Stransky<sup>2</sup> carried this study to rabbits and was unable to detect any choleretic effect of cinchophen in this species. Berman and Ivy<sup>3</sup> confirmed this finding and found in addition that the excretion of cinchophen in the bile of rabbits was very small.

Churchill and Van Wagoner<sup>4</sup> demonstrated that dogs receiving 20 to 30 times the human dose of cinchophen developed gastric or duodenal ulcers. This finding has since been confirmed very many times.<sup>5-7</sup> Shoji<sup>8</sup> found that rabbits were very resistant to the ulcero-

genic action of this drug. Okada<sup>9</sup> likewise failed to produce cinchophen ulcers in either rabbits or guinea pigs. Schwarz and Simmonds<sup>10</sup> were unable to induce cinchophen gastric, or duodenal ulceration in rabbits or guinea pigs, but found cats very susceptible. The ability of cinchophen to cause liver damage in rats<sup>11</sup> has been noticed but there has been no report of its effect upon the gastrointestinal tract.

<sup>5</sup> Stalker, L. K., Bollman, J. L., and Mann, F. C., *Arch. Surg.*, 1937, **34**, 1172.

<sup>6</sup> Bollman, J. L., and Mann, F. C., *Proc. Staff Mayo Clinic*, 1935, **10**, 580.

<sup>7</sup> Nasio, J., *Rev. Gastroenterol.*, 1946, **13**, 195.

<sup>8</sup> Shoji, A., *Trans. Soc. Path. Jap.*, 1933, **23**, 520.

<sup>9</sup> Okada, S., *Trans. Soc. Path. Jap.*, 1933, **23**, 522.

<sup>10</sup> Schwarz, S. O., and Simmonds, J. P., *Proc. Soc. Exp. Biol. and Med.*, 1935, **32**, 1133.

<sup>11</sup> Barbour, H. G., and Fisk, M. E., *J. Pharm. and Exp. Therap.*, 1933, **48**, 341.

<sup>1</sup> Brugsch, W., and Horsters, H., *Z. ges. Exp. Med.*, 1923, **38**, 367.

<sup>2</sup> Stransky, E., *Biochem. Z.*, 1925, **155**, 256.

<sup>3</sup> Berman, A. L., and Ivy, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 853.

<sup>4</sup> Churchill, T. P., and Van Wagoner, F. H., *Proc. Soc. Exp. Biol. and Med.*, 1931, **28**, 581.

TABLE I.  
Effect of Cinchophen Upon Bile Output in Various Animals and Its Excretion in the Bile.

No. of animals	Species	Avg wt, g	Cinchophen administered, mg/kg	Bile output				% recovery of cinchophen in bile in 1st 2 hr
				Control, ml in 30 min.	Control, ml/kg hr	1st 30 min. after cinchophen	Increase, %	
4	Rabbits	2,400	100	4.5	3.74	4.25	0	1.75
13	Rats	241	100	0.266	2.2	0.239	0	5.1
14	Guinea pigs	569	100	2.2	7.2	3.0	36.3*	27.7
11	Cats	2,850	100	0.77	0.54	0.95	23	2.18

\* This figure only is statistically significant ( $P = < 0.01$ ).

Except in the case of the rabbit and dog, it has not previously been shown whether or not any relationship exists between the susceptibility of an animal to cinchophen peptic ulcer and the animal's choleretic response to this drug.

*Methods.* Two series of experiments were conducted, acute experiments to determine the effect of cinchophen upon the bile flow and chronic feeding experiments to determine the susceptibility of the various animals to cinchophen ulceration.

*Acute experiments.* Cats, rabbits, guinea pigs and rats were used in this study. Dogs were not used as they have been adequately studied by others.<sup>1,12</sup> The animals having been fasted for the preceding 12 hours were anesthetized with "Nembutal". The gallbladder, where present, was tied off and the common duct cannulated using plastic tubing. In rats, a glass cannula of near-capillary dimensions attached by rubber tubing to a horizontal graduated 1 ml pipette was used. In the other species the bile was collected in 10 ml graduated cylinders. The volume was measured half-hourly. After a steady control output had been reached, cinchophen dissolved in — NaOH was administered intravenously. In those animals failing to respond, the reactivity was tested with sodium dehydrocholate ("Decholin"). Animals failing

to give a response to sodium dehydrocholate were discarded.

*Chronic Feeding Experiments.* In these studies the same four species were used with the addition of dogs. The latter were used to determine the ulcerogenic potency of the cinchophen. The drug was administered to cats, dogs, guinea pigs and rabbits daily in gelatin capsules. It was in addition administered subcutaneously to another series as an aqueous solution of the sodium salt to guinea pigs, rabbits and rats. To still another series of rabbits and rats a suspension of the free acid in tragacanth was administered by stomach tube. In these species this mode of administration was found more convenient than gelatin capsules. As it was observed that the animals most susceptible to cinchophen ulcer were carnivora, a series of rabbits, rats and guinea pigs was fed the same proprietary meat dog food as were the dogs and cats. In all cases control animals were fed the same diet without cinchophen as the test animals.

It is now well known that cholerisis results from the administration of cinchophen both

TABLE II.  
Apparent Relationship Between Cinchophen Excretion in Cats' Bile and Cholerisis Induced by Cinchophen.

Cat. no.	% cinchophen recovery in bile in 1st 2 hr.	% increase in bile flow
1	0.13	0
2	1.13	0
3	1.7	16.75
4	2.5	100
5	5.65	117.5

<sup>12</sup> Bradley, W. B., and Ivy, A. C., *Proc. Soc. Exp. Biol. and Med.*, 1940, 45, 143.

TABLE III.  
Incidence of Ulceration in Various Animals Resulting from Cinchophen Feeding.

No. of animals	Species	Dose range, mg/kg	Diet	Survival time, days	No. of animals showing	
					Ulcers	Erosions
21	Rabbits	100-600	Normal	12-32	0	0
11	"	100-200	Meat	12	0	2
20	Guinea	100-230	Normal	16.5	1	0
10	pigs	300	"	7.1	6	0
6	"	400	"	5	1	0
24	Rats	100-400	Meat and normal	S 42-56	0	0
11*	"	500 and 1500	Normal	S 15	2	5
12	"	600-700	"	S 10	10	1
6	Cats	100	"	9	3	1
4	"	200	"	6	3	0
1	Dogs	100	"	S 62	0	0
6	"	200	"	14	6	0

\* These animals were fed 500 mg/kg for 13 days and given 1,500 mg/kg on the 14th, and killed on the 15th.

S = Sacrificed.

by vein and orally to acute and chronic biliary fistula dogs.<sup>12</sup>

**Results.** It will be seen from Table I that guinea pigs show both the greatest choleretic response to cinchophen and also the greatest secretion of cinchophen in the bile. Rats and rabbits show no choleretic response. The rats, however, secreted appreciable quantities of cinchophen in the bile whereas the rabbits as was previously shown by Berman and Ivy<sup>3</sup> did not. Our results in cats were not clear cut. The average figures show a slight increase, but this was not statistically significant, many of the animals failed to respond to sodium dehydrocholate and were therefore not included in the results. It will be seen from Table II that the cinchophen output in the cats' bile shows individual variation, but nevertheless the magnitude of the choleretic effect appears to vary directly as the cinchophen output.

It was found (Table III) that among the species studied rabbits alone failed to develop cinchophen ulcers. Two gastric erosions occurred amongst the meat fed animals, but as these appeared with equal frequency in the controls fed meat without cinchophen the causative factor was thought to be the presence of fragments of bone in the meat.

The only ulceration seen in rats occurred in a series fed 500 mg/kg cinchophen daily for 13 days. On the 14th day, 1,500 mg/kg was administered. Two ulcers and a number of erosions (Table III) were observed at autopsy on the 15th day, and in another series in which 10 of one dozen rats, fed 600-700 mg/kg developed ulcers within 10 days (Fig. 1). The stomachs of all were full of food thus excluding starvation as a possible cause of the gastric lesions. All the ulcers seen occurred in the glandular part of the stomach. The meat diet seemed to be without effect.

Ulcers occurred in guinea pigs more frequently than in rats, but still with less frequency than in cats or dogs. They occurred only when doses in the neighborhood of 300 mg/kg were given daily. Above and below this dose level cinchophen killed the animals but ulcers were not seen. Meat feeding to guinea pigs over long periods of time was found impossible as the animals would starve rather than eat the diet. In our earlier investigation,<sup>13</sup> doses of 200 mg/kg were used and ulcers were not produced. A group of control animals fasted for one week without being given cinchophen developed the charac-

<sup>13</sup> Magee, D. F., Kim, K. S., and Ivy, A. C., *Fed. Proc.*, 1949, 8, 103.

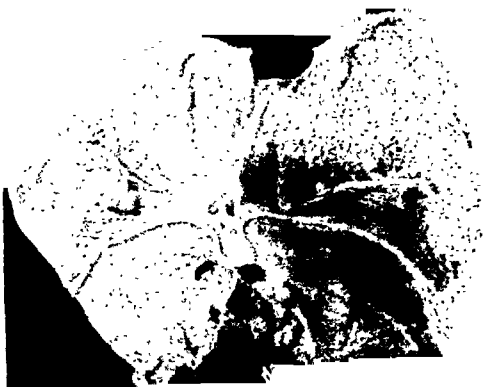


FIG. 1.

Ulcers produced in the rat's stomach by feeding 600 mg/kg cinchophen for 10 days.

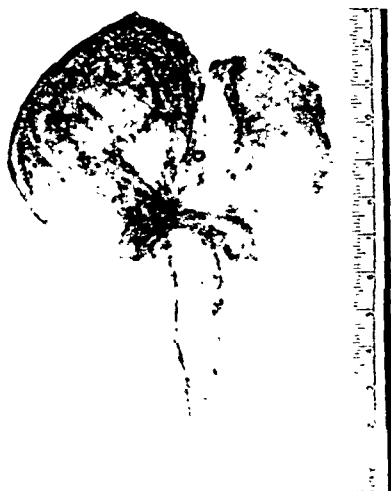


FIG. 2.

Typical gastric ulceration resulting from feeding 300 mg/kg cinchophen to guinea pigs.

teristic mottled yellow liver, seen in all animals except dogs, but no ulcers appeared. This excludes the possibility that cinchophen produced ulceration is secondary to loss of appetite and subsequent starvation.

The ulcers seen in guinea pigs were quite unlike those seen in other animals. They were almost always multiple and were confined almost exclusively to the fundic part of the stomach (Fig. 2) whereas in cats and dogs, in both of which ulcers occurred easily, they were pyloric, usually single, occasionally

3 or 4 were observed in cats. In guinea pigs there were seldom less than 20.

Similar ulcers were produced in guinea pigs using sodium dehydrocholate in doses of 200 mg/kg/day orally or subcutaneously often within 24 hours. Sellards<sup>14</sup> and Tsuruta<sup>15</sup> have produced this characteristic guinea pig type of ulceration with sodium glycocholate and the sodium salts of conjugated cholic acids obtained from ox bile.

*Discussion.* It appears, at least superficially, that cinchophen ulceration can be produced most easily in those species which show a cholerisis, but the choleretic response in cats to cinchophen is so small and variable as to be statistically insignificant, and moreover it is not certain that the ulcers in the guinea pig are of the same origin as those occurring in cats and dogs. Sodium dehydrocholate administered to guinea pigs will produce ulcers almost identical in site and appearance with those resulting from cinchophen. This has not been seen in the other animals. The ulcers produced in rats were in response to such enormous doses of cinchophen that a direct effect upon the mucosa cannot be excluded.

No correlation appears to exist between the cinchophen output in the bile and the species susceptibility to cinchophen ulceration. Rats and especially guinea pigs secrete very much more cinchophen in their bile than do cats and yet cats were the most ulcer susceptible animals studied.

The only factor which is common to all ulcer resisting species is that they have a high control output of bile. However, the most susceptible of the resisting species, the guinea pig, has the highest basal bile flow (Table I). Cats and dogs, both of which secrete approximately 0.5 ml per kg/hr are very susceptible. It is well known that dogs totally deprived of bile often develop spontaneous ulcers. A possibility which must be considered is that cinchophen has a different metabolic pathway in the more resistant group of animals from that in the less resistant species.

<sup>14</sup> Sellards, A. W., *Arch. Int. Med.*, 1909, 4, 502.

<sup>15</sup> Tsuruta, T., *Cincinnati Med. Bull.*, 1931, 6, 110.

*Summary and conclusions.* A choleric response to cinchophen was observed in guinea pigs.

The cinchophen output in the bile was found to be greatest in guinea pigs and to decrease in the following order: rats, cats and rabbits.

The following is the order found for susceptibility to cinchophen ulceration: cat, dog, guinea pig and rat. Rabbits were found completely resistant.

There appears to be no obvious relationship between the species susceptibility to cinchophen ulceration and the choleric response of that species to cinchophen or to the concentration of cinchophen in the bile after intravenous administration.

It would appear that the ulcer susceptible animals investigated have a low basal bile flow whilst the unsusceptible have a high flow.

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## Focal Neurological Lesions Produced by Microwave Irradiation. (17458)

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Prewar and wartime research allowed the development of electronic equipment capable of efficiently producing large quantities of radiation above 1000 megacycles (microwaves). Notable among these advances was the development of the magnetron tube which is used in radar equipment<sup>1</sup> and has been incorporated recently in a microwave diathermy machine.\* The frequency at which this machine operates (2450 megacycles, 12.2 cm) allows good penetration of animal tissue and even of bone, as demonstrated by Osborne and Fredericks<sup>2</sup> and Krusen, Herrick and Wakim.<sup>3</sup> These findings and the fact that radiation at this frequency travels in substantially straight lines suggests that the application of toxic doses of this radiation to a small area of the cerebral cortex is possible without injury to the scalp and skull.

However, a review of the literature reveals no report of the utilization of microwave radi-

ation for the artificial production of focal neurological lesions. With the technic to be described focal lesions were produced in rabbit brains without surgical procedure. The site of the cortical lesion, moreover, was accurately determined before the irradiation.

*Method.* Adult rabbits weighing about 3 kg were utilized. Under intravenous pentothal anesthesia sufficient to immobilize the animal (80-100 mg) the animal's head was placed on a wooden block approximately 7 cm in thickness. The area of the scalp over the proposed site of irradiation was shaved. A thin copper shield with an oval hole 2 x 3 cm was then placed with the long axis of the hole parallel to the rabbit and over the proposed site, in the present cases the right cerebral hemisphere. It was found convenient to braze a guide on the upper surface of the shield to maintain the intersection of the lead-in conductor and dipole directly over the hole in the shield. In the "C" type of director, which was used throughout, the point of maximum radiation was directly underneath the small screw which held the dipole in place.

The plastic protector of the radiating elements was then placed in contact with the shield and the shield in contact with the scalp over the proposed site. It was found convenient to mark the site lightly with a dot of colored crayon before putting the shield

<sup>1</sup> Argento, H. F., Centimeter-wave Magnetrons. QST, Dec. 1945.

\* Available as the "Microtherm" from the Raytheon Mfg. Co., Waltham, Mass., who courteously supplied the machine with which this work was done.

<sup>2</sup> Osborne, Stafford L., and Frederick, Jesse N., J.A.M.A., 1948.

<sup>3</sup> Krusen, F. H., Herrick, J. F., and Wakim, K. G., Proc. Staff Meet. Mayo Clin., 1947, 22, 209.



FIG. 1.

Circumscribed area of coagulation necrosis in the cerebral cortex of the rabbit. Nissl's stain. Very low power.

in place. The shield interferes considerably with the efficiency of the radiator but some shielding is necessary lest the ears be seriously burned, since they protrude directly beside the dipole. Prior to irradiation, 30 cc of 5% glucose in water and 100 mg caffeine sodium benzoate were injected intraperitoneally. With the output at 100% the machine was turned on for 3 minutes.

No quantitative temperature readings were taken. They were considered to be grossly inaccurate and misleading since the area of increased temperature within the intracranial cavity was so small.

*Results.* Seventeen rabbits were used but only 2 representative experiments will be described.

*Rabbit 18:* The animal received irradiation exactly as described above under "Method." He responded in 30 minutes after irradiation but refused to eat for three days. During this time he received 100 cc 5% glucose in water intraperitoneally daily. Following this he gradually rallied and took food spontaneously. At no time was an asymmetrical motor defect noted. Fifteen days after ir-

radiation the animal was living and well and showed only a crusting lesion of the scalp over the irradiated area. The animal was sacrificed on the 15th day by intracardiac ethyl alcohol. The brain grossly showed a yellow-green coagulation lesion 0.7x1 cm on the superior surface of the posterior portion of the right cerebral hemisphere. The dura was grossly intact and was not adherent to any area of the cortex. Histologically, as seen in Fig. 1, there was a small lesion which appeared sharply circumscribed. It involved the cortical gray matter in its entire width and extended somewhat into the subcortical white matter. The whole lesion contained no intact nerve cells or glia and was delimited by a wall of intensely proliferating glia cells.

*Rabbit 16:* This animal received irradiation according to the above technic but with elimination of the shield, intraperitoneal fluid and caffeine. The animal did not respond and expired three hours later despite post-irradiation parenteral fluids. Sections of the brain showed a large sloughed area with a slight polymorphonuclear infiltration in the surrounding surviving tissue.



*Comment.* It appears from the experiments reported that by means of microwave irradiations a focal lesion can be produced in the cerebral cortex of the rabbit without incising the scalp or opening the skull. The only injury to the scalp was a crusting lesion without bleb formation.

A possible explanation of the extremely small size of the lesion is that the plastic protector in contact with a small area of the scalp has dielectric properties which allow much more efficient entrance of the radiation into tissue in the area in contact, than is possible from air to tissue.

The histological lesions produced by this type of irradiation into the cerebral cortex are similar to those described in detail by Silver and Walker<sup>4</sup> in experiments of thermo-coagulation of the cerebral cortex by the direct application of a heated piece of metal, except that microwave destruction apparently penetrates more deeply within the tissue.

Since microwave diathermy heating is dielectric heating, it is presumed to be non-ionizing and any toxic effects would be those of excessive heating. In fact, no significant hematopoietic effects were observed in the work of Lidman and Cohen,<sup>5</sup> Fallis<sup>6</sup> and Daily<sup>7</sup> upon exposure to intensities of radia-

tion likely to be experienced in practical radar work. It should be noted, however, that Imig, Thomson, and Hines<sup>8</sup> indicate that testicular degeneration may occur from microwave heating at a lower temperature than from infra-red heating, and Richardson, Duane, and Hines<sup>9</sup> showed that upon intentional overdosage, lenticular opacities appeared at about 50°C.

Further work is now in progress using a special butyl rubber impregnated with a titanium compound which allows an intermediate dielectric constant without excessive absorption of the radiation by the rubber. With this technic it is hoped to allow penetration in an area of any shape and, because of the greater efficiency of transfer into the tissue, in a larger area. A modification of this technic may eventually allow the easy destruction of cortical tissue without any surgical procedure both in experimental neurology and in therapeutics.

*Summary.* A technic is described of producing focal lesions in the cerebral cortex of rabbits by microwave irradiation without incising the scalp or skull. The results on two animals are reported and future possibilities of this work are discussed.

<sup>7</sup> Daily, L. E., *U. S. Naval Med. Bull.*, 1943, **41**, 1052.

<sup>8</sup> Imig, C. J., Thomson, J. D., and Hines, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 383.

<sup>9</sup> Richardson, A. W., Duane, T. D., and Hines, H. M., *J. Neuropath. Exp. Neur.*, 1947, **10**, 311.

Received October 18, 1949. P.S.E.B.M., 1949, **72**.

<sup>4</sup> Silver, M. L., Walker, A. E., *J. Neuropath. Exp. Neur.*, 1947, **10**, 311.

<sup>5</sup> Lidman, B. I., and Cohen, C., *Air Surg. Bull.*, 1945, **2**, 448.

<sup>6</sup> Fallis, R. H., Jr., *Am. J. Physiol.*, 1946, **147**, 281.

## Ultrafiltration and Ultracentrifugation Studies of Coxsackie Virus. (17459)

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The physical properties of the Coxsackie group of viruses<sup>1-4</sup> have been under investiga-

tion since their isolation, and preliminary studies indicated that the original strain is very small. The present report is a summary

<sup>1</sup> Dalldorf, Gilbert, and Sickles, G. M., *Science*, 1948, **108**, 61.

<sup>2</sup> Dalldorf, Gilbert, Sickles, G. M., Plager, Hildegard, and Gifford, Rebecca, *J. Exp. Med.*, 1949, **89**, 567.

<sup>3</sup> Gifford, Rebecca, and Dalldorf, Gilbert, *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 589.

<sup>4</sup> Sickles, G. M., and Dalldorf, Gilbert, *Proc. Soc. Exp. Biol. and Med.*, 1949, **72**, 30.

of the observations based on filtration through gradocol membranes prepared according to Elford's technic and on sedimentation in the ultracentrifuge. The methods of Bauer and Hughes<sup>5</sup> were used in the filtration experiments, the membranes being sterilized by ultraviolet light. The ultracentrifuge is of the Beams-Pickels type with a rotor of 6-inch diameter. The original strain, T.T., of Cocksackie virus, serologic type 1,<sup>4</sup> was employed in the present work. The legs of infected immature mice and hamsters and the brains of immature mice have served as sources of the virus. The titer of virus in the extremities is markedly higher than that in the central nervous system.<sup>3</sup> For comparison, similar studies were made with brain suspensions of mice infected with MM virus.

*Size of Cocksackie Virus.* Ten-percent suspensions of the legs of immature hamsters infected with Cocksackie virus and 10% suspensions of MM-infected-mouse brains were prepared in 0.85% salt solution containing 10% infusion broth at pH 7.4. After clarification by centrifugation in a Sorvall angle centrifuge at 8,000 r.p.m. (about 6000 g) for 30 minutes and filtration through a Seitz E-K pad, the suspensions were ultrafiltered on 39  $\mu$ , 33  $\mu$ , and 18  $\mu$  gradocol membranes. The Cocksackie virus suspension, initially having an infectivity titer of approximately  $10^6$ , passed all 3 membranes. Similar results were secured with other Cocksackie virus suspensions prepared from mouse skeletal tissues and brains. Application of Elford's correction factor would indicate that the particle size is 10  $\mu$  or less.

MM virus suspensions similarly tested and treated passed the 39  $\mu$  and 33  $\mu$  membranes and were completely retained by the 22  $\mu$  and 18  $\mu$  membranes. The size of the MM virus estimated from these results would lie between 11 and 16  $\mu$ ,

which agrees with results reported by other workers<sup>6,7</sup> and indicates that the membranes were satisfactory.

*Purification.* Suspensions of both viruses have been subjected to ultracentrifugation. It has been reported by Melnick, Shaw, and Curnen<sup>8</sup> that the Cocksackie virus could be largely precipitated after spinning at 31,200 r.p.m. (about 110,000 g) for one hour. In our experience with Cocksackie and MM viruses, much, but not all, of the virus appears in the clear brown pellets. Both viruses have also been purified by the use of methanol according to the method of Pollard.<sup>9</sup> We have used 60% methanol as a precipitating agent and a final concentration of 25%. A 0.2 M phosphate buffer at pH 8.0 was used for elution. Satisfactory results were obtained with either virus present as a suspension of infected mouse brain. The recovery of Cocksackie virus from infected immature mouse legs was less satisfactory, perhaps because of the fat present in these suspensions. It is noteworthy that Elford's<sup>10</sup> results indicate that the virus of Newcastle disease is not readily recovered with 20% ethanol at 0°C.

*Summary.* The original strain of Cocksackie virus, serologic type 1, has been measured using Elford's ultrafiltration technic. Ultracentrifugation furnished a rough check. The virus is very small, the estimated size being 10  $\mu$  or slightly less.

<sup>6</sup> Jungeblut, C. W., and Dalldorf, Gilbert. *Am. J. Pub. Health*, 1943, **33**, 169.

<sup>7</sup> Gollan, Frank. *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 364.

<sup>8</sup> Melnick, J. L., Shaw, E. W., and Curnen, E. W., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 344.

<sup>9</sup> Pollard, Morris, Connolly, Joan, and Fromm, Stanley. *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 290.

<sup>10</sup> Elford, W. J., Chu, C. M., Dawson, I. M., Dudgeon, J. A., Fulton, F., and Smiles, J., *British J. Exp. Path.*, 1948, **20**, 590.

<sup>5</sup> Bauer, J. H., and Hughes, T. P., *J. Gen. Physiol.*, 1934, **18**, 143.

## Effects of Administration of Testosterone on Vitamin A-Deficient Rats. (17460)

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It has been shown by Mason<sup>1</sup> in particular that the effect of vitamin A deficiency on the male rat amounts to a virtual castration. This phenomenon can lead to two main hypotheses. One, vitamin A deficiency may interfere either with the synthesis of testosterone, directly (chemically) or indirectly (e.g. secondary effect due to pituitary atrophy in deficient animals). The second probability is that the response of target organs to circulating male sex hormones is affected. To elucidate this point, it was decided to examine and compare the effect of testosterone administration on castrated and non-castrated vitamin A deficient male rats and their controls.

**Method.** The animals used were male Wistar albino weanlings, 23 days of age. Their average weight was 55 g. Castrated animals had been operated at the age of 21 days and subsequent autopsies showed that all operations had been successful. Prior to the weaning of the experimental animals, their mothers had been put on a vitamin A-poor, "depletion" diet. The animals were kept at room temperature (about 70° F) and were alternately 12 hours in darkness and 12 hours in artificial light. They were fed water and food *ad libitum*. The diet used was the U.S.P. XII diet for vitamin A assay (casein 18%, salt mixture 4%, yeast 8%, starch 65% and vegetable oil 5%) to which was added 100 mg per kg of  $\alpha$ -tocopherol. With a yeast-containing diet and a slow growing strain of animals, secondary vitamin C deficiency does not develop as a complicating factor.<sup>2,3</sup> Control animals received orally 1200 I.U. vitamin A every 8 days.\*

The animals receiving testosterone were injected every day subcutaneously with 2 mg

of testosterone propionate ("Perandren") dissolved in 0.04 cc of sesame oil.<sup>†</sup> Other animals received a control daily injection of 0.04 cc of pure sesame oil. Thirty-one animals were used. They were divided into the following 8 groups:

- a) 6 non-castrates, vit. A deficient
- b) 6 non-castrates, vit. A deficient receiving testosterone
- c) 3 non-castrates receiving vit. A
- d) 3 non-castrates receiving vit. A and testosterone
- e) 4 castrates, vit. A deficient
- f) 4 castrates, vit. A deficient receiving testosterone
- g) 3 castrates, receiving vit. A
- h) 2 non-castrates receiving vit. A and testosterone

All animals were weighed every other day. They were sacrificed by decapitation after they had been 64 days on the experiment. By that time most deficient animals showed ocular symptoms of vitamin A deficiency and had lost some weight.

**Results and discussion.** Table I gives the average weight gains as well as significant average organ weights found. As most groups of animals were too small to permit legitimate statistical treatment and comparison, Table II gives the values of all ratios —

organ weight  
body weight

found so as to show the distribution of results.

Selye<sup>4</sup> conducted a systematic study of the

\* The authors are indebted to Dr. E. L. Sevringhaus, medical director, Hoffmann-LaRoche, Nutley, N. J., for gifts of vitamins.

† The authors are indebted to Dr. Roy Hertz and to Dr. K. W. Thompson of the Roche-Organon Co. for gifts of "Perandren."

<sup>4</sup> Selye, H., *Proc. Soc. Exp. Biol. and Med.*, 1941, **46**, 142.

<sup>1</sup> Mason, K. E., *Am. J. Anat.*, 1933, **52**, 153.

<sup>2</sup> Mayer, J., and Krehl, W., *Arch. Biochem.*, 1949, in press.

<sup>3</sup> Mayer, J., *Rev. Can. Biol.*, 1949, in press.

TABLE I.  
Effect on Body and Organ Weight. Gain in weight during experimental period.

	a. No vit. A No testost.	b. No vit. A + testost.	c. + vit. A No testost.	d. + vit. A + testost.
1. Non-castrates				
Final* wt gain	44 g	44 g	104 g	84 g
Max. wt gain	54 g	50 g	id.	id.
Testes*	1068 mg	932 mg	2247 mg	1833 mg
Seminal vesicles†	107 mg	1727 mg	193 mg	1749 mg
Kidneys	1149 mg	1448 mg	1769 mg	2187 mg
	e. No vit. A No testost.	f. No vit. A + testost.	g. + vit. A No testost.	h. + vit. A + testost.
2. Castrates				
Final wt gain	46 g	47 g	104 g	105 g
Max. wt gain	52 g	57 g	id.	id.
Seminal vesicles†	— ‡	1417 mg	— ‡	1720 mg
Kidneys	1083 mg	1560 mg	1517 mg	2429 mg

\* The difference between "Final" and "Maximum" gain weights is due to the fact that when the animals were sacrificed, the degree of deficiency reached was such that most animals had already lost weight.

† Prostates and epididymes showed size changes in the same directions as seminal vesicles.

‡ Not detectable by macroscopic examination.

effects of various doses of testosterone on the weight of the body and of certain organs of the male albino rat. He found particularly striking effects for doses of 1.0 to 10 mg daily. This is an analogous dosage to that used here. The experiment was continued for only 20 days—approximately one-third of the duration of the present experiment. In these conditions, he observed that accessory sex organs, the seminal vesicles in particular were very much enlarged, while the size of the testes was decreased. In this particular series of his no significant effect was observed on the weight of the kidneys, in contradistinction with some other of his own observations<sup>5-7</sup> and those of other investigators.<sup>8-10</sup>

In the present work, the deficient animals, castrates as well as non castrates conformed to the general pattern established and verified for the control animals: testosterone administration was followed by extreme hypertrophy of accessory sex organs, seminal vesicles in

particular; by a definite hypertrophy of the kidneys and by a decrease of the size of the testes. As far as the effect on the overall body weight is concerned, while the decrease in weight gain of normal animals under the influence of testosterone administration noted by Selye was confirmed, deficient animals behaved like castrates in that they were not affected.

Particularly significant is the fact that the extreme hypertrophy of accessory sex organs was maintained by testosterone even in the face of acute deficiency. Animals showing marked periocular symptoms and which had sustained severe losses of weight extending over a period of two weeks still showed seminal vesicles, prostates and epididymes 10 times larger, in absolute values, than control animals not receiving testosterone. This differential reaction is in itself quite striking.

This experiment seems to establish definitely the fact that any "castration effect" observed as a result of vitamin A deficiency in the rat is not due to the lack of response of target organs but to lack of circulating androgens. Because of previous results indicating the apparent "piling up" of cholesterol, in Vitamin A deficiency,<sup>11-13</sup> a possible explanation

<sup>5</sup> Selye, H., *J. Urol.*, 1939, **42**, 637.

<sup>6</sup> Selye, H., *Canad. Med. Assn. J.*, 1940, **42**, 113.

<sup>7</sup> Selye, H., *J. Endocrinol.*, 1939, **1**, 208.

<sup>8</sup> Korenchevsky, V., and Ross, M. A., *Brit. Med. J.*, 1940, **1**, 645.

<sup>9</sup> Paschkis, K. E., Shay, H., Gershon-Cohn, J., and Fels, S. S., *Am. J. Physiol.*, 1940, **129**, 191.

<sup>10</sup> Pfeiffer, C. A., Emmel, V. M., and Gardner, W. U., *Yale J. Biol. Med.*, 1940, **12**, 493.

<sup>11</sup> Ralli, E. P., and Waterhouse, A., *Proc. Soc. Exp. Biol. and Med.*, 1933, **30**, 519.

TABLE II.

Organ weight

Relationship. Distribution of Values.

Body weight

		a. No vit. A No testost.		b. No vit. A + testost.		c. + vit. A No testost.		d. + vit. A + testost.	
Non-castrates									
Testes		10.4	11.5	8.5	10.8	14.3	14.2	12.9	13.4
$\times 10^3$		11.2	9.8	8.5	12.2	14.4		14	
body		10.2	12.8	10.8	8.5				
		Avg 11		Avg 9.9		Avg 14.3		Avg 13.4	
Seminal vesicles		0.002	1.78	15	18.4	1.7	0.8	10	12.5
$\times 10^3$		0.64	0.8	17.2	23.2	1.4		17.8	
body		2	1.47	17.7	18.8				
		Avg 1.1		Avg 18.4		Avg 1.2		Avg 13.4	
Kidneys		14.7	15	13.6	15	13.4	9.8	16.3	16
$\times 10^3$		13.8	12.9	19.8	14.8	10.5		14.3	
body		13.3	12.2	13.4	13.2				
		Avg 13.7		Avg 15.0		Avg 11.2		Avg 15.6	
		e. No vit. A No testost.		f. No vit. A + testost.		g. + vit. A No testost.		h. + vit. A + testost.	
Castrates									
Seminal vesicles		—		12.9	12.5	—		10.3	10.9
$\times 10^3$				13.1	12.7			Avg 10.6	
body				Avg 12.8					
Kidneys		12.1	12.8	14	14.2	9.3	9.2	14.2	15.8
$\times 10^3$		14.1	13.4	14.1	14.4	9.5			
body		Avg 13.1		Avg 14.2		Avg 9.3		Avg 15.0	

tion is that vitamin A deficiency prevents chemical transformation of cholesterol into testosterone. Indirect mechanisms, such as intervention of vitamin A in the synthesis or release of gonadotrophic hormones cannot, however, be eliminated without further experimental work.

*Summary.* 1. In an effort to find whether the "virtual castration" of vit. A deficient male rats is due to prevention of the synthesis of androgens or to the lack of response of target organs, the effects of administration of testo-

sterone in deficient non-castrates and castrates were compared with the effects on their controls.

2. The response of the deficient animals was found to be essentially normal. The accessory sex organs in particular were extraordinarily hypertrophied under the influence of testosterone administration even in the face of severe losses of weight.

3. The evidence obtained suggests that vitamin A deficiency interferes with the synthesis or release of androgens.

<sup>12</sup> Smith, M. E., *J. Nutrition*, 1934, **8**, 675.

<sup>13</sup> Mayer, J., unpublished observations.

## Blood Histamine, Leukocytes and Platelets in Experimental Serum Disease in Rabbits. (17461)

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The role of histamine in allergic diseases is poorly understood. During anaphylactic shock the blood histamine level has been found to be markedly elevated in dogs and guinea pigs immediately following the reinjection of antigen.<sup>1-5</sup> It is believed that this elevation can account for practically all dominant symptoms of this condition except the incoagulability of the blood which is explained by outpouring of heparin.<sup>5</sup> However, in rabbits, horses, and calves the blood histamine level has been shown to be depressed.<sup>5,6</sup> In rabbits this deviation can plausibly be explained by liberation of histamine from the buffy coat which in these animals contains 200  $\gamma$  of histamine per 100 cc of blood as compared with 2 to 4  $\gamma$  in dogs and 12 to 25  $\gamma$  in guinea pigs.<sup>7</sup> The depression of the blood histamine in horses and calves is difficult to explain on this basis for these animals contain only 2 to 4  $\gamma$  of histamine per 100 cc of blood.

The role of histamine in serum disease has been studied by several investigators. Dammin and Bukantz<sup>7</sup> were unable to produce the vascular lesions of this disease in normal rabbits by giving them daily intravenous injections of 0.1 mg of histamine base per kg of body weight for periods varying from 1 to 62 days. Injecting histamine into the rabbit's skin seldom caused erythema or whealing, and the site of injection, unlike that of an Arthus

phenomenon, generally did not fluoresce following the intravenous injection of fluorescein. Moreover, in rabbits injected with horse serum, neohetramine given subcutaneously in doses of 10 mg per kg twice daily did not affect the appearance of circulating antibodies, the Arthus reaction of the skin or the development of arteritis.<sup>7</sup> Benadryl in doses of 5 mg per kg every 8 hrs was likewise ineffective.<sup>8</sup>

However, the latter drug in doses of 2 or 10 mg twice daily, and drug 1627, another antihistaminic agent, have been reported to impede the development of the myocardial lesions of serum disease,<sup>9</sup> and benadryl in doses of 5 mg per kg every 8 hrs apparently offered some protection against the production of lesions in the valves.<sup>8</sup> These results have not been duplicated.<sup>7,8</sup> Antistine has been found to prevent experimental glomerulonephritis in rabbits produced by anti-kidney serum,<sup>10</sup> but the glomerulitis of experimental serum disease was not affected by antihistaminics.<sup>11</sup>

In view of these various findings it seemed of interest to study the blood histamine level in experimental serum disease, and as the histamine of the blood is known to be contained in the buffy coat, to investigate the leukocytes and platelets as well.

**Material and Methods.** All experiments were performed in Chinchilla rabbits averaging 1900 g in weight. Twenty-five rabbits were given 15 cc per kg of "normal horse serum" (Squibb) containing 1:10000 sodium ethyl mercuri thiosalicylate plus 0.2% phenol. The remaining 15 rabbits received horse

<sup>1</sup> Dragstedt, C. A., and Mead, F. B., *J. Immun.*, 1936, **30**, 319.

<sup>2</sup> Dragstedt, C. A., and Mead, F. B., *J. Pharm. and Exp. Therap.*, 1936, **57**, 419.

<sup>3</sup> Simon, A., and Staub, A. M., *Compt. rend. Soc. de biol.*, 1937, **125**, 815.

<sup>4</sup> Code, C. F., *Am. J. Physiol.*, 1939, **127**, 78.

<sup>5</sup> Dragstedt, C. A., *Physiol. Rev.*, 1941, **21**, 563.

<sup>6</sup> Code, C. F., and Hester, H. R., *Am. J. Physiol.*, 1939, **127**, 71.

<sup>7</sup> Dammin, G. J., and Bukantz, S. C., *J. Am. Med. Assn.*, 1949, **139**, 358.

<sup>8</sup> Roberts, R. C., Crockett, K. A., and Laipply, T. C., *Arch. Int. Med.*, 1949, **83**, 48.

<sup>9</sup> Kyser, F. A., McCarter, J. C., and Stengle, J., *J. Lab. and Clin. Med.*, 1947, **32**, 379.

<sup>10</sup> Reubi, F., *Helvet. Med. Acta*, Suppl. 18, 1946.

<sup>11</sup> Janeway, C. A., oral communication, 1949.

serum without preservative (Wyeth).<sup>\*</sup> Seventeen days later the surviving animals were desensitized with 1 cc of serum intravenously and 2 days thereafter they were reinjected with another 15 cc per kg. Twenty-four rabbits received serum only, 16 were given 20 mg of neohetramine in a 4% solution subcutaneously 4 times daily (at 9 AM and at 1, 5 and 9 PM). The injections of the drug were discontinued 3 days after reinjection of the serum, *i.e.*, 4 days before the last 4 rabbits receiving the drug were sacrificed. Histamine was determined according to Code's method.<sup>12</sup> The blood was collected from the heart in quantities of 10 cc. In order to avoid a possible change in the blood histamine level by repeated bleeding, blood was drawn as a rule only once or twice from the same animal except in a last series of 3 rabbits which were bled 5 times each. The dates of collection are indicated in Fig. 1. Leukocyte and platelet counts were first done on certain days only, but in our last series of 3 rabbits 12 determinations were made in each animal. All counts were made shortly before blood was collected for histamine determination. They were made on blood obtained by nicking the marginal vein of the ear with a sharp razor blade. Groups of 4 animals were sacrificed and studied microscopically 3, 4, 5, 7, 14 and 16 days after the first injection of serum, and 2, 6 and 7 days after the second.

**Results.** The Arthus reaction of the skin elicited by intradermal injection of 0.1 cc of horse serum was, as in the experiments of Dammin and Bukantz,<sup>7</sup> not appreciably changed by neohetramine in 20 rabbits tested. Analysis of the urine at the time of sacrifice showed no significant difference between the rabbits receiving neohetramine and the controls during the later stages of the experiment. However, the proteinuria which occurred during the week following the first injection of

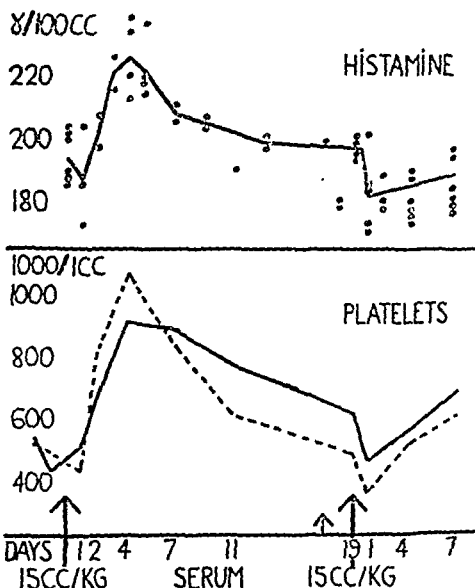


FIG. 1.

Histamine and platelet levels in serum disease. The large arrows indicate the first and second injection of 15 cc of horse serum per kg, the small arrow the injection of the desensitizing dose of 1 cc.

horse serum was abolished by the antihistaminic (Table I). The histamine level of the blood before injection averaged 194  $\gamma$  per 100 cc in 6 rabbits tested. After the intravenous injection of 15 cc of horse serum per kg the level rose to an average of 226  $\gamma$  on the 4th day and then gradually returned to normal (Fig. 1). Reinjection of serum on the 19th day was followed by a sharp drop in histamine to an average of 182  $\gamma$  on the 1st day. There was no elevation of this material during the first 7 days after the second injection.

The rabbits which received neohetramine revealed an average blood histamine level of

TABLE I.  
Protein Content of Urine Following the First Injection of Serum.

Days after inj.	Without Neohetramine	With
4	+	0
	+ - +	0
5	+	0
	+	0
7	(+)	(+)
	(+)	0
14	(+)	0
	0	0

<sup>\*</sup> We are indebted to Dr. J. Seifter of the Wyeth Institute of Applied Biochemistry for supplying us with the neohetramine solution, and Dr. B. Scott Fritz and Dr. J. H. Brown of the Wyeth Incorporated Biological Laboratory at Marietta, Pa., for giving us the horse serum.

<sup>12</sup> Code, C. F., *J. Physiol.*, 1937, **89**, 257.

TABLE II.  
Leukocyte and Platelet Levels Following the First and Second Injection of Serum in Rabbits No. 1, 2, 3.

Days after inj. Rabbit No.	Neutrophiles			Lymphoid cells			Eosinophiles			Basophiles			Platelets (thousands)		
	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
	Before inj.														
	1622	2967	2342	4400	3349	4314	31	0	0	187	64	136	543	524	756
	1228	1872	2630	4182	4017	4558	30	30	37	140	121	185	427	499	685
	After first inj.														
1	1408	988	2442	4847	2660	4514	131	95	74	164	57	370	511	428	533
2	4493	1084	1588	7128	3729	4027	60	25	0	300	202	265	660	817	475
4	3640	6837	3220	3100	4596	3187	0	0	0	0	57	133	908	1068	701
7	2784	3559	4232	5193	5703	3976	42	49	0	291	439	342	898	850	936
11	3353	2550	5408	3724	3858	7452	112	0	0	261	302	330	774	610	670
19	5490*	1404	1350	5270*	3591	7850	0	27	0	330	378	800	613	493	1064
	After second inj.														
1	4085*	1564	7709	2074	1717	2399	31	17	0	0	102	102	458	357	727
4	1952	1748	2138	8991	7343	3680	59	50	0	828	849	1192	562	524	669
7	1639	2275	2984	6423	4302	3581	89	71	37	709	462	858	682	609	790

\* This elevation is accounted for by a retro-aural abscess which developed in this rabbit.

73  $\gamma$  per 100 cc (42 to 102) from the 3rd to the 17th day after the first injection, and 72  $\gamma$  after the second. The blood for these determinations was drawn mostly 4 hrs after the last injection of neohetramine.

The results of the leukocyte and platelet counts in the 3 most completely studied rabbits are given in Table II and Fig. 1. It is apparent that the first injection of serum caused first a rise in eosinophiles (on the 1st day) which was followed by a rise in neutrophils (between the 2nd and 4th day) and lymphoid cells (on the 4th day) whereas the basophiles were not much changed. While 2 of the rabbits behaved much alike with the exception of a high neutrophile count on the 19th and 20th day in Rabbit 1 (caused by a retro-aural abscess), and while most of our counts in other rabbits fell well within the range of the counts of these 2 animals, Rabbit 3 behaved differently showing a peak in neutrophils on the 11th day and one in lymphoid cells between the 11th and 19th day. At autopsy this animal revealed the most severe arteritis of all animals of this series: it also showed a marked glomerulitis though this was less severe than that in Rabbit 2.

The reinjection of serum on the 19th day caused a marked drop in lymphoid cells and basophiles one day after injection. This was followed by a marked rise of the 2 with a

peak on the 4th day. The average rise in lymphocytes was 178% (161% over the normal control counts), that in basophiles 1300% (590% over the normal control counts). The neutrophils showed no consistent change.

The platelets were markedly elevated following the first injection of serum with a peak on the 4th day: they showed a marked drop one day after the second injection and thereafter returned to normal (Fig. 1). Rabbit 3 differed again from this course, whereas most of our counts in other rabbits fell well within the range of counts of Rabbits 1 and 2. The course of the platelets closely paralleled that of histamine.

The important morphological changes in our rabbits are given in Table III. It can be seen that the rabbits which were treated with serum containing preservative showed more pulmonary involvement and less myocarditis, glomerulitis and arteritis than those receiving serum without preservative. This difference may have been due to precipitation and therefore increased retention in the pulmonary vessels of the preservative containing serum. For sections of these lungs showed numerous emboli blocking arterioles and capillaries of the lungs, whereas those of animals receiving serum without preservative did not. The chief difference in the reactions toward the



TABLE III.  
Frequency (a)\* and Intensity (b)† of the Various Lesions Caused by Serum.

No. of inj. of serum	Neohetramine	No. of rabbits	Mesenchymal myocardium		Reactions, lungs		Glomerulitis		Valvulitis		Arteritis	
			a.	b.	a.	b.	a.	b.	a.	b.	a.	b.
Serum with preservative												
1	with	8	62.5	52.5	100	48.5	0	0	0	0	0	0
	without	8	50	52.5	100	83	12.5	17	0	0	0	0
2	with	3	100	57.5	100	100	33.3	33	66.7	25	0	0
	without	3	100	62.5	100	92	33.3	17	50	100	33.3	50
	" ‡	6	100	75	100	71	66.7	21	40	83	16.7	50
Serum without preservative												
1	with	3	100	21	66.7	31	0	0	0	0	0	0
	without	4	100	44	100	34.5	25	17	0	0	0	0
2	with	2	100	75	100	50	100	54	100	83	50	50
	without	2	100	31	100	56	50	50	—	—	50	75
	" ‡	6	100	39.5	100	71	83.3	67	40	67	50	67

\* The frequency has been expressed in per cent of animals affected in order to facilitate comparison.

† The intensity has been expressed in per cent of the maximum intensity observed. The figures were calculated from the positive cases only.

‡ These animals were members of separate groups not receiving neohetramine.

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Neohetramine was found to depress the mesenchymal reactions in lungs and myocardium. This effect was most noticeable in the organs whose mesenchyme reacted most severely to the serum, *i.e.*, in the lungs, in the rabbits treated with serum containing preservative, and in the lungs and myocardium, in the animals which were given serum without preservative. Also the 2 cases of glomerulitis observed after the first injection of serum occurred both in animals without neohetramine. Following the 2nd injection no significant difference was observed between the two series

of experiments. However, the antihistaminic drug was discontinued 4 days before the last animals were sacrificed.

**Discussion.** The first intravenous injection of a large dose of horse serum in our rabbits caused a rise in histamine and platelets, the peak occurring on the 4th day. The reinjection of the same quantity of the same serum 19 days later was followed by a marked drop of the two elements during the first day, and there was no rise above normal during the remaining 6 days of the experiments. The rise in platelets after the first injection resembled that following various infections, the exposure to ultraviolet light, and other injuries,<sup>16</sup> while the drop in histamine after reinjection was in accord with the observed fall of histamine in anaphylactic shock in rabbits.<sup>5</sup> The rise in platelets following the first injection and the absence of a rise after reinjection furnish an explanation for the recent observation of increased blood coagulability during the first week following the first injection of serum and its absence after the second.<sup>17</sup> The close parallel between histamine and platelets in our rabbits (Fig. 1) is in keeping with the demonstration by Minard<sup>18</sup> and others that in rabbits most of the blood histamine is contained

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<sup>15</sup> Forman, C., Seifter, J., and Ehrlich, W. E., *J. Allergy*, 1949, **20**, 273.

<sup>16</sup> Gunn, F. D., *Arch. Path.*, 1931, **12**, 153.

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It is obvious that the rise in histamine and platelets following the first injection of serum cannot be regarded as an allergic phenomenon; it is apparently part of the primary reaction of the organism against noxious agents in general. During the period of histamine and platelet elevation following the first injection of serum all rabbits tested which received serum only contained protein in the urine, whereas, those which were given neohetramine did not. Though one may be tempted to explain this as a specific antihistaminic effect, there is reason to believe that this was due to a non-specific action of the drug upon the permeability of the capillaries possibly via the hyaluronidase-hyaluronic acid mechanism; for it has been found that antihistaminic drugs decrease the permeability of tissue membranes *in vitro*<sup>18</sup> as well as that of capillaries *in vivo*,<sup>20,21</sup> and it has also been observed that hyaluronidase enhances the leakage of protein through the glomeruli in serum disease in rabbits.<sup>22</sup> However this may be, microscopic examination of the kidneys showed that the proteinuria in our animals was not caused by proliferative glomerulitis, but it was associated with degenerative changes such as swelling of the loops and pyknosis of the nuclei as described and illustrated in a previous paper.<sup>14</sup>

The mesenchymal reactions in the lungs following the first injection of serum were distinctly, and those of the myocardium slightly depressed by neohetramine. The latter observation is in accord with the findings of Kyser and coworkers.<sup>9</sup> The depressing effect of neohetramine, if real, is opposite to that of hyaluronidase<sup>22</sup> and therefore may well be explained by a non-specific action of this drug on the permeability of tissue membranes causing diminished penetration of lungs and

myocardium by antigen. The development of arteritis, glomerulitis and valvulitis following reinjection, on the other hand, was not affected. These observations are in keeping with our view that the mesenchymal reactions in lungs and myocardium are direct reactions to the antigen instrumental in the production of antibody, whereas arteritis and glomerulitis are the result of an antigen-antibody reaction.<sup>14,15</sup>

Of the changes of the leucocytes, the marked rise in basophiles following the second injection of serum is of particular interest. As the reinjection is followed by outpouring of heparin,<sup>5</sup> and there is reason to believe that the basophiles are cellular sources of heparin,<sup>23</sup> it suggests itself that the rise in basophiles may be a regenerative phenomenon prompted by the heparin consuming effect of the anaphylactic reaction.

**Conclusions.** It appears that in rabbits histamine is linked via the histamine containing platelets with blood coagulation, and this is connected via heparin with the heparin producing basophiles. All these elements are involved in serum disease. The first injection of serum is followed by a rise in histamine and platelets, the second by a drop in these elements. The destruction of the white cell layer following reinjection calls forth an outpouring of heparin which in turn is followed by basophilia in the blood. Only the changes following reinjection can be regarded as allergic. Neohetramine was found to effect only the primary reactions following the first administration of serum. It suppressed the rise in blood histamine and the proteinuria observed during the first week following the first injection, and also impeded the primary mesenchymal reactions in lungs and heart which are believed to be instrumental in antibody formation. There are reasons to believe that this was not a specific antihistaminic effect, but that it was due to a non-specific action of the drug upon the permeability of tissue membranes possibly via the hyaluronidase-hyaluronic acid mechanism.

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<sup>19</sup> Seifter, J., Baeder, D. H., and Dervinis, A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **72**, 136.

<sup>20</sup> McGavack, T. H., Elias, H., and Boyd, L. J., *Am. J. Med. Sc.*, 1947, **213**, 418.

<sup>21</sup> Leger, J., and Masson, G., *ibid.*, 1947, **214**, 305.

<sup>22</sup> Seifter, J., and Ehrlich, W. E., to be published.

<sup>23</sup> Jorpes, J. E., Heparin, Oxford University Press, 1946.

TABLE III.  
Frequency (a)\* and Intensity (b)† of the Various Lesions Caused by Serum.

No. of inj. of serum	Neohetramine	No. of rabbits	Mesenchymal myocardium		Reactions, lungs		Glomerulitis		Valvulitis		Arteritis	
			a.	b.	a.	b.	a.	b.	a.	b.	a.	b.
Serum with preservative												
1	with	8	62.5	52.5	100	48.5	0	0	0	0	0	0
	without	8	50	52.5	100	83	12.5	17	0	0	0	0
2	with	3	100	57.5	100	100	33.3	33	66.7	25	0	0
	without	3	100	62.5	100	92	33.3	17	50	100	33.3	50
	" ‡	6	100	75	100	71	66.7	21	40	83	16.7	50
Serum without preservative												
1	with	3	100	21	66.7	31	0	0	0	0	0	0
	without	4	100	44	100	34.5	25	17	0	0	0	0
2	with	2	100	75	100	50	100	54	100	83	50	50
	without	2	100	31	100	56	50	50	—	—	50	75
	" ‡	6	100	39.5	100	71	83.3	67	40	67	50	67

\* The frequency has been expressed in per cent of animals affected in order to facilitate comparison.

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<sup>23</sup> Jorpes, J. E., Heparin. Oxford University Press, 1946.

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<sup>19</sup> Seifter, J., Baeder, D. H., and Dervinis, A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **72**, 136.

<sup>20</sup> McGavack, T. H., Elias, H., and Boyd, L. J., *Am. J. Med. Sc.*, 1947, **213**, 418.

<sup>21</sup> Leger, J., and Masson, G., *ibid.*, 1947, **214**, 305.

<sup>22</sup> Seifter, J., and Ehrlich, W. E., to be published.

# Detoxification of Cottonseed Pigment Glands with Ferrous Sulfate. (17462)

EDWARD EAGLE (Introduced by A. J. Carlson)

From the Research Laboratories, Swift and Co., Chicago, Ill.

The use of iron salts as antidotes for cottonseed meal injury was suggested many years ago by Withers *et al.*<sup>1</sup> who successfully fed ferric ammonium citrate-treated cottonseed meal to rabbits and ferrous sulfate and ferric chloride-treated meal to pigs. In a recent paper<sup>2</sup> we studied the acute oral toxicity on rats, mice, rabbits and guinea pigs of cottonseed pigment glands, distinct morphological structures recently made available from cottonseed kernels by a flotation process.<sup>3</sup> The present report concerns LD50 studies on rats of different samples of pigment glands each of which was administered in both distilled water and in 2% ferrous sulfate solution.

**Procedure.** Male rats weighing between

150 and 220 g were fasted for 18 hours (average weight loss from fasting was 14 g). Water was given *ad libitum*. Each rat was kept in a separate cage in an air-conditioned animal room maintained at  $80^{\circ} \pm 1^{\circ}\text{F}$  and ca. 45% relative humidity. For each of the 3 samples the test preparations were made both by mixing 10 g of the pigment glands with distilled water to a final volume of 100 ml, and by suspending 10 g of the same glands in 2% ferrous sulfate solution to a final volume of 100 ml. Single doses were given by stomach tube on the basis of milligrams of pigment glands per kilogram body weight. All animals had free access to stock diet and water after dosing. Calculations of the LD50

TABLE I.  
Mortality in Rats after Oral Administration of Single Doses of Cottonseed Pigment Glands in Water and in Ferrous Sulfate Solution.

Dose, mg/kg	Sample 1		Sample 2		Sample 3		
	In water	In 2% FeSO <sub>4</sub>	In water	In 2% FeSO <sub>4</sub>	In water	In 2% FeSO <sub>4</sub>	In 0.5% FeSO <sub>4</sub>
7000		0/4		0/4		0/1	4/4
6000		0/4		0/4		0/4	4/4
5400				0/4		0/4	
5000		0/4		0/4		0/4	4/4
4600					4/4	0/4	
4200					5/7	0/4	
4000		0/4		0/4	3/4		4/4
3800					6/7	0/4	
3400		1/4		0/4	4/4	0/5	4/4
3000		0/4		0/4	8/11	0/5	4/4
2600	3/3	0/4	4/4		3/3		
2400			4/4				
2200	3/4	0/4	4/4		6/11		
2000			4/4				
1800	7/7	0/4	3/4		4/4		
1600			3/4				
1400	7/8		2/4		0/8		
1200	2/4		0/4				
1000	3/7						
Total rats used	33	36	32	28	63	36	24
LD50 (mg/kg)	1140	>7000	1490	>7000	2290	>7000	<3000

<sup>1</sup> Withers, W. A., and Brewster, J. F., *J. Biol. Chem.*, 1913, **15**, 161; Withers, W. A., and Caruth, F. E., *ibid.*, 1917, **32**, 245.

<sup>2</sup> Eagle, E., Castillon, L. E., Hall, C. M., and Boatner, C. H., *Arch. Biochem.*, 1948, **18**, 271.

<sup>3</sup> Boatner, C. H., and Hall, C. M., *Oil and Soap*, 1946, **23**, 123; Vix, H. L. E., Spadaro, J. J., Westbrook, R. D., Croveto, A. J., Pollard, E. F., and Gastroek, E. A., *J. Am. Oil Chemists' Soc.*, 1947, **24**, 228.

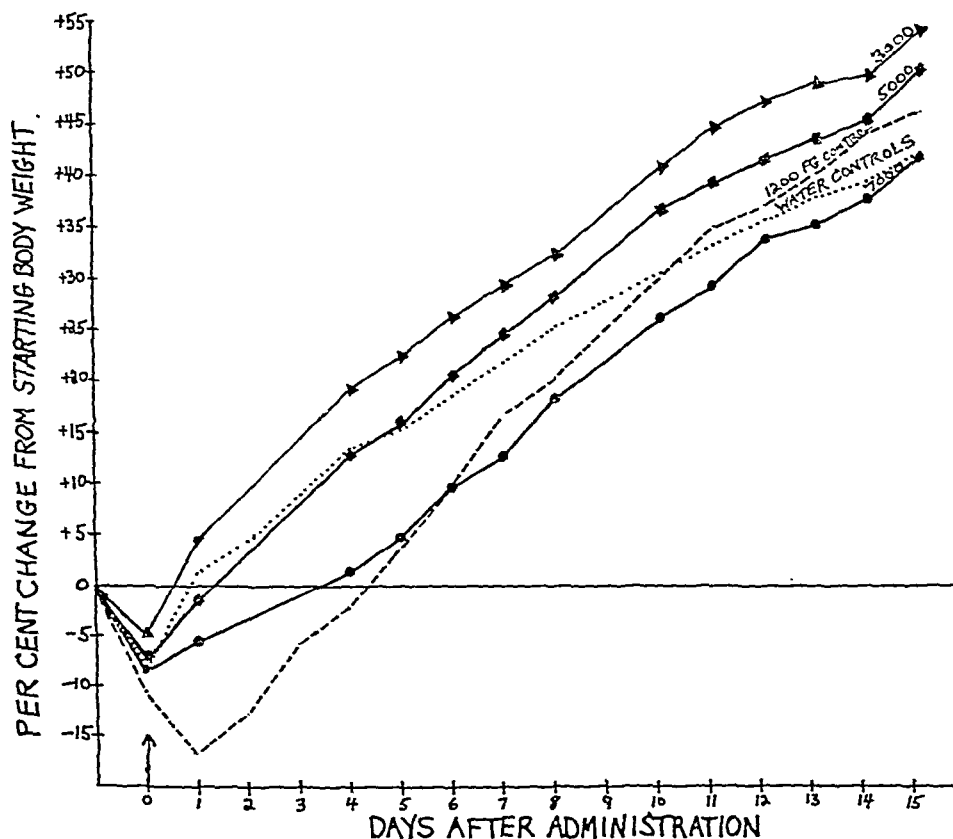


FIG. 1.

Effect on the body weight of the rat of large, single doses of cottonseed pigment glands detoxified by administration in 2% ferrous sulfate solution.

values were made by the method of Reed and Muench.<sup>4</sup>

**Results.** A summary of the experimental data is given in Table I. Doses of all 3 samples of cottonseed pigment glands which caused 100% mortality when administered in distilled water had no harmful effect when given in 2% ferrous sulfate solution. In the case of Sample 3, cottonseed pigment glands which were completely detoxified when given in 2% FeSO<sub>4</sub> lost none of their toxicity when suspended in 0.5% FeSO<sub>4</sub> and all of the rats died. The rapid onset of diarrhea reported for cottonseed pigment glands<sup>2</sup> occurred at every dose level when distilled water was the vehicle, but no diarrhea occurred when the glands were suspended in 2% ferrous sulfate solution. All

fatalities except one occurred within 3 days, 82% within 24 hours. Typical body weight curves obtained on rats after administration of unusually large single doses (indicated by arrow) of cottonseed pigment glands (Sample 2) are illustrated in Fig. 1. Each point (solid lines) represents an average value obtained from 4 rats. The water control rats (dotted line) received water only; the pigment gland control rats (dashes) received 1200 mg/kg of the glands suspended in water. Since the LD<sub>50</sub> value for these pigment glands was 1490 mg/kg, all of the experimental rats would have died had these large doses (3000 to 7000 mg/kg) been administered in distilled water instead of in 2% FeSO<sub>4</sub> solution. The 1200 mg/kg dose of pigment glands in water caused a marked depression in body weight; the large doses administered in 2% FeSO<sub>4</sub> caused no decreased weight beyond the

<sup>4</sup> Reed, L. J., and Muench, H., *Am. J. Hygiene*, 1938, 27, 493.

fasting level. Similar body weight curves were obtained with 22 other dose levels of the 3 samples of cottonseed pigment glands.

*Summary.* Acute toxicity studies were made on 3 different samples of cottonseed pigment glands administered orally to 228 rats in the form of slurries made with either distilled water or 2% ferrous sulfate solution. The LD50 values for the samples when administered in water were, respectively, 1140, 1490 and 2290 mg/kg. When given in 2% ferrous sulfate solution the samples were detoxified so that even such high doses as 7000 mg/kg for each were no longer toxic. There was little

effect on the body weight of all 99 rats which survived doses of ferrous sulfate-treated cottonseed pigment glands 3 to 6 times the LD50 value for water-suspended glands. Administration of pigment glands in 0.5% FeSO<sub>4</sub> did not detoxify them, and led to the death of all 24 rats given this preparation.

The author is indebted to Mr. H. F. Bialek for technical assistance, and to the Southern Regional Laboratory, U.S.D.A., New Orleans, La., for the samples of cottonseed pigment glands.

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### Effect of 3-Ortho-toloxo-1,2-propanediol (Tolserol, Myanesin) upon Electrogram of Human Cortex and Subcortex. (17463)

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The sites of action of 3-ortho-toloxo-1,2-propanediol (tolserol, myanesin) have not been exactly determined as yet, although there is suggestive evidence that this drug chiefly affects subcortical ganglia (Berger and Bradley;<sup>1</sup> Burke and Linegar;<sup>2</sup> Davison;<sup>3</sup> Pugh and Enderby;<sup>4</sup> Schlesinger *et al.*<sup>5</sup>). Particularly the experience that it is able to abolish petit mal discharges (Gammon and Churchill<sup>6</sup>) points to a thalamic effect, since these discharges seem to originate in thalamic nuclei (animal experiments of Jasper,<sup>7</sup> human records of Wycis, Lee and Spiegel<sup>8</sup>).

A study of the effect upon the electrical

discharges of subcortical ganglia promised to supply more direct evidence. In the course of thalamotomy and mesencephalotomy operations carried out by means of our stereoencephalotome (Spiegel and Wycis<sup>9</sup>), we had an opportunity to record the potentials of human diencephalic and mesencephalic structures preceding the production of the lesions.

The effect of tolserol upon the electroencephalogram (scalp electrodes in the frontal and in the occipital region), upon the electrical discharges of thalamic nuclei (dorso-medial nucleus; lateral nucleus), of the hypothalamus, and of the tectum of the midbrain was studied. Needle electrodes consisting of an inner stylet, insulated except for the tip, and an outer sheath insulated except for a ring 3.0 mm above the tip of the stylet were inserted by means of the stereoencephalotome. Monopolar as well as bipolar leads were used. For monopolar recording, the tip of the inner

<sup>1</sup> Berger, F. M., and Bradley, W., *Brit. J. Pharm.*, 1946, 1, 265.

<sup>2</sup> Burke, J. C., and Linegar, C. R., *Fed. Proc.*, 1948, 7, 208.

<sup>3</sup> Davison, W. H. A., *Brit. Med. J.*, 1948, 1, 544.

<sup>4</sup> Pugh, J. I., and Enderby, G. E. H., *Lancet*, 1947, 2, 387.

<sup>5</sup> Schlesinger, E. B., Drew, A. L., and Wood, B., *Am. J. Med.*, 1948, 4, 365.

<sup>6</sup> Gammon, G. D., and Churchill, J. A., *Am. J. Med. Sc.*, 1949, 217, 143.

<sup>7</sup> Jasper, H. H., and Droogleever-Fortuyn, J., *Res. Publ. Assn. Nerv. Ment. Dis.*, 1947, 26, 272.

<sup>8</sup> Wycis, H. T., Lee, A. J., and Spiegel, E. A., *Conf. Neur.*, 1949, 9, 264.

<sup>9</sup> Spiegel, E. A., Wycis, H. T., Freed, H., and Lee, A. J., *Proc. Soc. Exp. Biol. and Med.*, 1948, 69, 175.

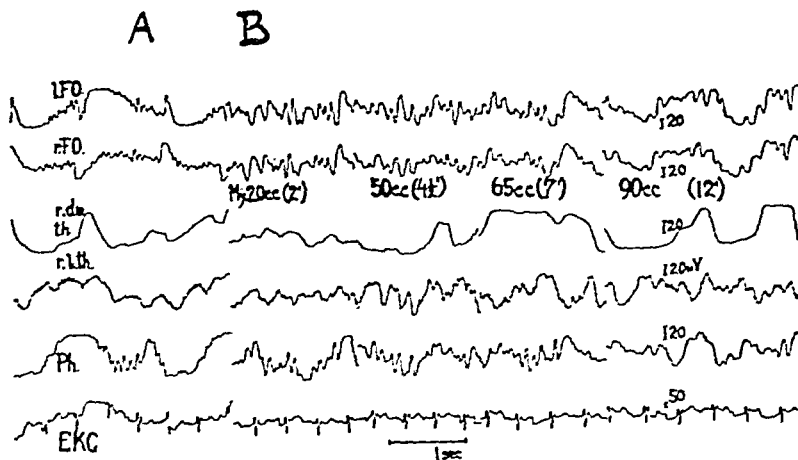


FIG. 1.

Effect of intravenous injection of 2% Tolserol solution in a schizophrenic patient chiefly upon the EEG (1FO bipolar record from left frontal and occipital scalp electrodes, rFO bipolar record from right frontal and occipital scalp electrodes). No definite effect upon the dorsomedial nucleus (r. dm. th) and the lateral nucleus (r l th) of the right thalamus. No definite changes in the pharyngogram (Ph) and the electrocardiogram (EKG). A large part of the right dorsomedial nucleus is degenerated by a previous thalamotomy. A before, B after injection of Tolserol (Myanesin My). Time after injection is indicated in brackets. Anesthesia: .6 mg Atropine sulf.; 30 cc Paraldehyde: Demerol 37.5 mg; 0.5 g Sod. Pentothal;  $N_2O-O_2$  (3:1).

stylet served as the active, the lobe of the homolateral ear as the "indifferent" electrode. For bipolar recording the tip of the stylet served as the one and the bare ring on the sheath as the 2nd electrode. Simultaneously the electrocardiogram and the electrical discharges lead off from the roof of the pharynx were recorded. These records were obtained in schizophrenic patients, cases of depression and obsessive compulsive neuroses preceding thalamotomy and in cases of unbearable pain preceding mesencephalotomy. All patients were under nembutal or pentothal,  $N_2O-O_2$  anaesthesia; 50-100 cc 2% Tolserol\* were injected intravenously.

The following changes of the electrical activity were observed: In some instances, the fast, low voltage oscillations of the EEG of the anesthetized patients were replaced by slower waves of higher amplitude (Fig. 1). The speed of the injection seems of importance, if one wishes to demonstrate alterations of the EEG. We observed the above described effect, if the first 50 cc of the drug were

injected within 4-5 minutes and the following 50 cc within 6-8 minutes. In contrast to the susceptibility of the cerebral cortex to tolserol, we failed to obtain definite changes of the EEG following the injection of curare in doses sufficient to produce peripheral paralysis. This latter observation is in agreement with the findings of Harvey,<sup>10</sup> Everett,<sup>11</sup> and Girden.<sup>12</sup> While no definite changes in the discharges of the dorsomedial nuclei (dm th) and of the lateral nuclei (lat th) of the thalamus were found (Fig. 1), the hypothalamic lead (Hm and Hb) showed a definite depression of the discharges interrupted by the appearance of burst of slow waves of high amplitude (Fig. 2). These changes appeared at a stage when a cortical effect was not definite as yet. The same applies to the tectum of the midbrain whose fast, low voltage discharges were replaced by high 3-5/second waves (Fig. 3).

**Summary:** Tolserol has a definite effect

<sup>10</sup> Harvey, A. M., *Fed. Proc.*, 1948, **7**, 458.

\* Kindly supplied by Dr. H. Sidney Newcomer, E. R. Squibb and Sons.

<sup>11</sup> Everett, G. M., *J. Pharm. and Exp. Therap.*, 1948, **92**, 246.

<sup>12</sup> Girden, E., *J. Neurophysiol.*, 1948, **11**, 169.



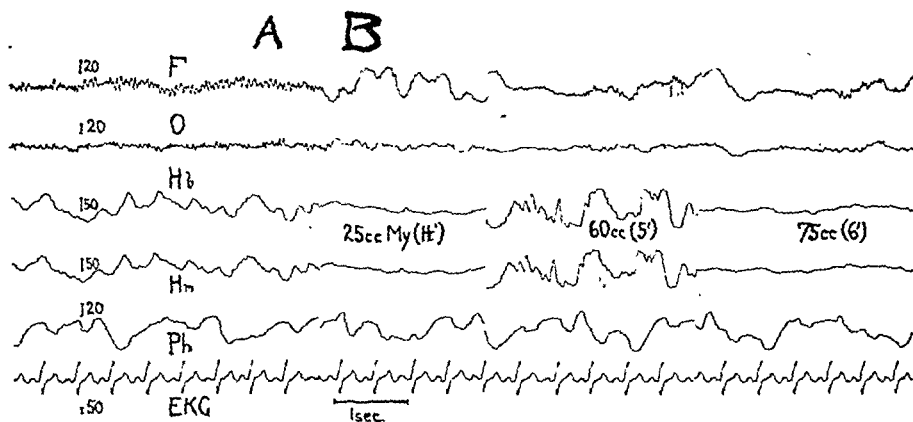


FIG. 2.

Effect of intravenous injection of 2% Tolserol solution (My) chiefly upon the hypothalamogram in a paranoid schizophrenic. Hb bipolar, Hm monopolar records from the hypothalamus. Note that the pharyngogram does not reflect the changes in the hypothalamogram. F bipolar record from the frontal areas, O from the occipital areas (scalp electrodes) A before, B after injection of the drug. Anesthesia: Atropine sulf. 0.4 mg; Sod. Phenobarbital 0.2 g; Sod. Pentothal 1.3 g; Curare 100 units;  $N_2O-O_2$ .

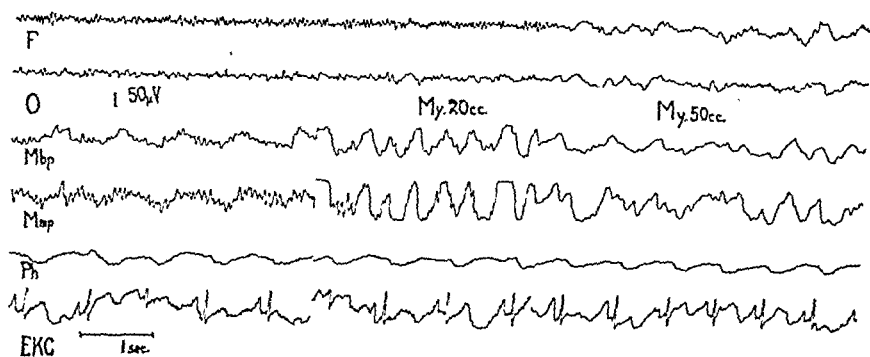


FIG. 3.

Effect of intravenous injection of 2% Tolserol solution (My) upon the tectum mesencephali in a case of severe pain. Mbp bipolar, Mmp monopolar records from the tectum of the mid-brain. F bipolar frontal, O bipolar occipital records (scalp electrodes). A before, B after injection of the drug. Anesthesia: Atropine sulf. 0.6 mg; Nembutal 200 mg; Curare 100 units; Sod. Pentothal 0.375 g;  $N_2O-O_2$ .

upon the electrical discharges of the human cerebrum; the most pronounced changes were found in the electrical activity of the hypothalamus and of the midbrain.

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# Further Studies on the Histochemical Specificity of Phosphatases.\* (17464)

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In continuation of previous studies,<sup>1</sup> 10 more substrates were tried in histochemical experiments on phosphatases. The new substrates were the following: catechol- and hydroquinone monophosphates; glucose-6-phosphate, fructose-6-phosphate, phosphoglyceric acid; yeast adenylic acid (3-nucleotide), muscle adenylic acid (5-nucleotide); dimethyl- and diphenyl acid pyrophosphates, and casein. The first two were prepared by a modification of the King-Nicholson<sup>2</sup> procedure; instead of the Ba salts the Ca salts were prepared; the crude products were purified by extraction with water at an alkaline reaction and precipitation by 1 to 2 volumes of alcohol. Glucose-6-phosphate was the gift of Dr. B. Vennesland of the Dept. of Biochemistry, University of Chicago; fructose-6-phosphate, phosphoglyceric acid and yeast adenylic acid were obtained from the Schwarz Laboratories, New York; 5-nucleotide was purchased from the Sigma Chemical Co., St. Louis; dimethyl acid pyrophosphate was received from the Victor Chemical Works, Chicago; diphenyl acid pyrophosphate was synthesized according to the method of Neuberg and Wagner.<sup>3</sup>

**Experimental.** The technic was the same as reported previously. The substrates were used at two pH values only, at pH 5 and 9.2. Slides incubated with glycerophosphate were used as standards for comparison at both pH values. No reaction was obtained with casein in any of the tissues either at alkaline or at acid reaction.

In the alkaline range, intense reactions were

observed in 2 hours with all other substrates, except the pyrophosphates which required prolonged incubation (up to 24 hours). The picture of distribution of enzymatic activity was essentially identical with all substrates, except 5-nucleotide. Minor and mostly inconstant differences were noted with the pyrophosphates, the nuclei being stained relatively more intensely than with the use of the other substrates.

Pictures obtained with the use of 5-nucleotide were different in several respects from those observed with any of the other substrates. Although in some of the organs (kidney, adrenal, intestinal mucosa, embryonic skeleton, placenta, etc.) the pattern of enzymatic distribution was indistinguishable from that seen with the use of, *e.g.*, glycerophosphate, in other organs patterns entirely different and specific for 5-nucleotide were found. Some of the main differences were as follows:

1. In the brain of the rat and mouse, non-specific reaction is found mainly in the grey cortex where the reaction is rather diffuse, and in the walls of blood vessels (Fig. 1). With 5-nucleotide, the cortex is only slightly stained, and the reaction is mainly in the axons. Intense staining is obtained at some sites where the nonspecific reaction is practically negative such as the caudate nucleus (Fig. 2), the radiation of the corpus callosum and the

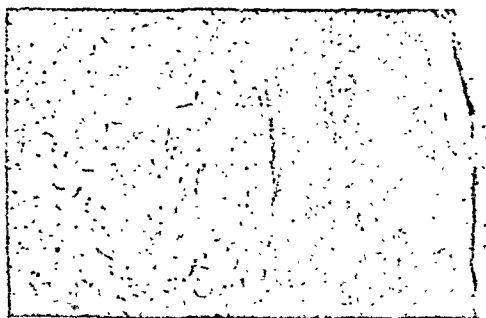


Fig. 1.  
Brain of the mouse. Substrate: glycerophosphate. Grey cortex and blood vessels stained.

\* This work has been done under grants from the Douglas Smith Foundation for Medical Research of The University of Chicago, and from the Pathology Study Section of the U. S. Public Health Service.

<sup>1</sup> Gomori, G., *PROC. SOC. EXP. BIOL. AND MED.*, 1949, **70**, 7.

<sup>2</sup> King, E. J., and Nicholson, T. F., *Biochem. J.*, 1939, **33**, 1182.

<sup>3</sup> Neuberg, C., and Wagner, J., *Biochem. Z.*, 1926, **171**, 485.



FIG. 2.

Another section of the same block. Substrate: 5-nucleotide. Caudate nucleus intensely stained.

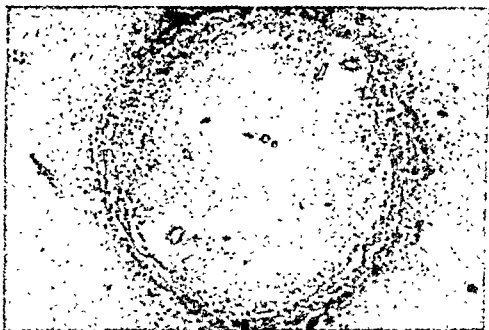


FIG. 3.

Human spleen. Substrate: glycerophosphate. Positive reaction at the periphery of a Malpighian body.



FIG. 4.

Consecutive serial section to the previous one. Substrate: 5-nucleotide. Reaction throughout the Malpighian body.

structures of the cornu Ammonis. The blood vessels are less intensely stained.

2. In the human spleen, only the periphery of the Malpighian bodies shows a nonspecific

activity (Fig. 3); with 5-nucleotide, the centers are also intensely stained (Fig. 4).

3. In the testis of man, the mouse and the rat, 5-nucleotidase activity is quite intense in the nuclei of spermatogenic elements while nonspecific activity is much weaker.

4. Smooth muscle of vessel walls and of some viscera (urinary bladder, stomach, etc.) stains intensely although neither uniformly nor consistently when the substrate is 5-nucleotide, while the nonspecific reaction is negligible in the same locations. On the other hand, longitudinal muscle of the uterus of the mouse stains equally well with all substrates.

These findings seem to confirm previous contentions<sup>4,5,6</sup> that 5-nucleotidase is a specific enzyme, different from nonspecific alkaline phosphatase. Whether the multiple overlapping of patterns of distribution is genuine or in part due to impurities of substrate nature in the muscle adenylic acid used in these experiments will require further investigation.

In the acid range, good results were obtained with catechol monophosphate and with glucose- and fructose-6-phosphates; with the other substrates the reactions were much weaker and variable. No significant differences in localization of activity were seen with different substrates, except that phenylpyrophosphate tended to show a predominantly nuclear pattern of activity. The findings of Reis<sup>7</sup> according to which prostatic acid phosphatase is also primarily a 5-nucleotidase could not be confirmed histochemically since the intensity of the reaction was far more marked either with glycerophosphate or with 3-nucleotide than with 5-nucleotide as a substrate.

**Summary.** The pattern of distribution of alkaline phosphatase activity in tissues is, when 5-nucleotide is used as a substrate, different from patterns obtained with any other substrate. The specificity of 5-nucleotidase appears to be an established fact.

<sup>4</sup> Reis, J., *Bull. soc. chim. biol.*, 1934, **16**, 385.

<sup>5</sup> Reis, J., *Enzymologia*, 1937-38, **2**, 110.

<sup>6</sup> Gulland, J. M., and Jackson, E. M., *Biochem. J.*, 1938, **32**, 590, 597.

<sup>7</sup> Reis, J., *Enzymologia*, 1938, **5**, 251.

## Absorption and Excretion of Streptomycin Para-aminosalicylate.\* (17465)

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(Introduced by C. C. Sturgis)

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University Hospital, Ann Arbor.*

Streptomycin has become firmly established as a valuable agent in the treatment of selected cases of human tuberculosis. Para-aminosalicylic acid (hereafter designated PAS) is effective in the treatment of experimental tuberculosis in animals<sup>1</sup> and appears to enhance the effectiveness of streptomycin when it is used in combined therapy in experimental infections.<sup>2</sup> Recent evidence from clinical trials<sup>3,4</sup> suggests that a combination of parenteral streptomycin and oral PAS acts to delay the accumulation of strains of tubercle bacilli resistant to either drug.

Basic streptomycin can be combined with 3 molecules of PAS to form streptomycin para-aminosalicylate<sup>5</sup> (hereafter designated streptomycin PAS): one gram of streptomycin combining with 0.8 g of PAS. The resulting chemical is very slightly, if at all, more irritating when injected intramuscularly than is an equivalent amount of streptomycin. It has been found to be superior to streptomycin in experimental tuberculosis in the mouse;<sup>5</sup> a daily dose of streptomycin PAS, equivalent to 0.4 mg of streptomycin affording the same

degree of protection as that produced by 2 to 3 mg of streptomycin sulphate. The present report concerns the results of investigation into the fate of streptomycin PAS in human subjects.

*Materials and methods.* The subjects were young patients with minimal or moderately advanced pulmonary tuberculosis as the only significant deviation from normality. Streptomycin PAS was injected intramuscularly in quantities equivalent to 1.0 g of streptomycin (0.8 g of PAS) in 4 subjects, and 0.5 g of streptomycin (0.4 g of PAS) in 4 other subjects. Plasma specimens (heparinized) were obtained for control purposes, and at 15 minutes, 30 minutes, and 1, 2, 4, 8 and 12 hours following injection of the material. Urine was collected in 3 pooled specimens each covering a 4 hour period. For purposes of comparison 2 of the above subjects were given an amount of PAS (0.8 g) orally equivalent to that contained in the streptomycin PAS which they had received by intramuscular injection in a previous experiment. Two subjects were given streptomycin PAS orally and in this case plasma specimens were collected only at 30, 60 and 120 minutes. Concentrations of PAS in plasma were determined by the method described by Marshall<sup>6</sup> using sodium para-aminosalicylate as standard material for calibration of the Evelyn colorimeter. Streptomycin concentrations were measured by the cup-plate method of bioassay in use in this laboratory. According to custom, concentrations of PAS are expressed as mg per 100 ml while concentrations of streptomycin are expressed as  $\gamma$  per ml. The control plasma in most individuals contains interfering substances which give readings for PAS estimated as near, but slightly less than 0.05 mg per 100 ml. This figure has, there-

\* This investigation was supported, in part, by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service.

<sup>†</sup> Charles Pfizer Fellow in Chemotherapy.

<sup>1</sup> Feldman, W. H., Karlson, A. G., and Hinshaw, H. C., *Proc. Staff Meet., Mayo Clin.*, 1947, **22**, 473.

<sup>2</sup> Youmans, G. P., Youmans, A. S., and Osborne, R. R., *Journal-Lancet*, 1947, **67**, 403.

<sup>3</sup> Karlson, A. G., Pfuertze, K. H., Carr, D. T., Feldman, W. H., and Hinshaw, H. C., *Proc. Staff Meet., Mayo Clin.*, 1949, **24**, 85.

<sup>4</sup> Delaude, A., Karlson, A. G., Carr, D. T., Feldman, W. H., and Pfuertze, K. H., *Proc. Staff Meet., Mayo Clin.*, 1949, **24**, 341.

<sup>5</sup> Prepared and furnished by the Charles Pfizer Company, Brooklyn, N. Y.

<sup>6</sup> Hobby, G. L., and Lenert, T. F., Presented at the 7th Streptomycin Conference, Veterans Administration, Denver, April 1949.

<sup>6</sup> Marshall, E. K., Jr., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 471.

fore, been accepted as a baseline. The addition of streptomycin PAS to water or serum gives recoveries, by the above methods, very near the expected values for both PAS and streptomycin. This would appear to indicate that the chemical union does not interfere with the biologic activity of streptomycin nor with the chemical determination of PAS.

**Results and discussion.** The plasma streptomycin concentrations, measured in the subjects who received streptomycin PAS intramuscularly, varied but were within the range expected for an equivalent amount of streptomycin. The values obtained for plasma PAS likewise varied considerably, particularly in the early minutes of absorption. Peak values developed between 15 and 30 minutes after intramuscular injection and averaged 1.35 mg per 100 ml in the subjects receiving an amount of streptomycin PAS equivalent to 1.0 g of streptomycin. In those subjects receiving a dose one-half this size the peak plasma concentrations averaged 0.6 mg per 100 ml. Concentrations fell rapidly and were at the base line after 4 hours in the group receiving the smaller dose and only very slightly above the base line in those who received the larger amount. This data is illustrated in Fig. 1. In Fig. 2, the curve of plasma PAS following ingestion of 0.8 g of PAS is compared with that obtained, in the same individual, following intramuscular administration of an equivalent amount of PAS as streptomycin PAS. The curves are very similar save that the peak concentration appeared later when the material was taken orally. Virtually the same relationships were observed in a second individual.

One fairly consistent feature of these experiments was the disassociation between the curves for PAS and for streptomycin concentrations in plasma. Peak levels for PAS appeared between 15 and 30 minutes while those for streptomycin occurred from 1 to 2 hours after injection (Fig. 3). Presumably this is due to rapid conjugation of the PAS radical<sup>1</sup> although it may be due, at least in part, to

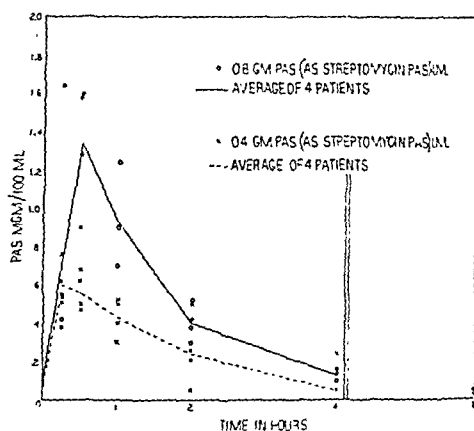


FIG. 1.

Plasma concentrations of PAS following intramuscular administration of streptomycin PAS in amounts equivalent to 1 g of streptomycin (0.8 g PAS) and 0.5 g streptomycin (0.4 g PAS). The lines indicate average values for each dosage level.

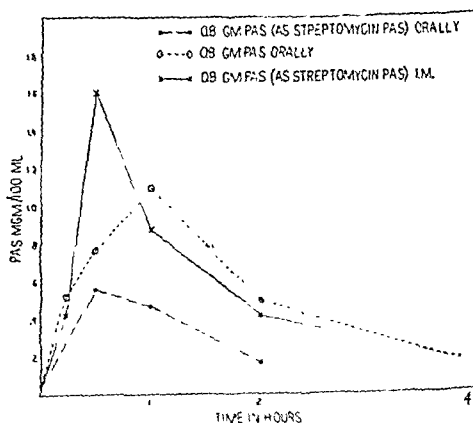


FIG. 2.

Comparison of plasma concentrations of PAS following equivalent amounts of: (1) Streptomycin PAS intramuscularly. (2) Streptomycin PAS orally. (3) PAS orally.

breakdown of the compound followed by independent behavior of streptomycin and PAS or its derivatives. Calculations of the expected levels of PAS, based on the concentrations of streptomycin in the plasma, were often fairly close to the observed levels of PAS for specimens taken 15 minutes after injection of the material. Later specimens usually exhibited PAS levels far below the calculated values, which might be expected if the PAS were undergoing rapid conjugation.

<sup>1</sup> Way, E. L., Smith, P. K., Howie, D. L., Weiss, R., and Swanson, R., *J. Pharm. and Exp. Therap.*, 1948, 93, 369.

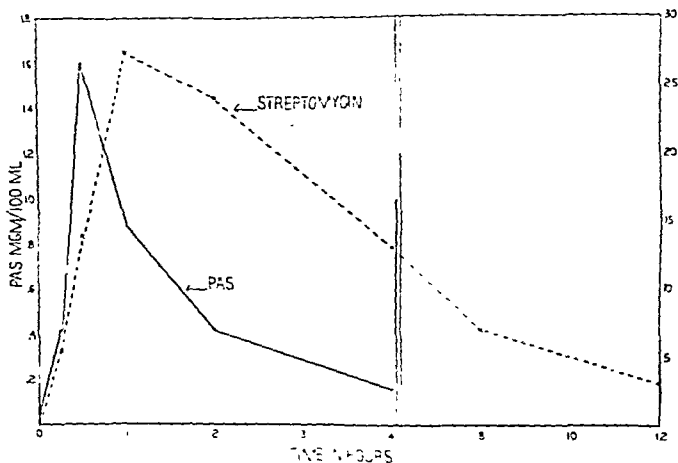


FIG. 3.

Plasma values for PAS and for streptomycin following intramuscular administration of an amount of streptomycin PAS equivalent to 1 g of streptomycin.

Streptomycin could not be detected in the plasma following oral administration of streptomycin PAS. The concentrations of PAS which developed were considerably lower than those expected from an equivalent amount of PAS, taken by mouth, or injected intramuscularly as streptomycin PAS, indicating that the material is certainly broken down to its components in the gastro-intestinal tract, though possibly not completely.

Urinary recovery of PAS following intramuscular administration of streptomycin PAS ranged from 9 to 24% (average 14%) with virtually the entire amount appearing within 4 hours of administration. Recovery of streptomycin averaged the expected 60%.

The concentrations of PAS in plasma following intramuscular administration of streptomycin PAS are not particularly impressive when compared with those following large amounts of PAS given orally (peak concentrations of 4 to 8 mg per 100 ml following doses of 3 g). They assume somewhat greater

significance, however, when considered in relation to the sensitivity of newly isolated strains of tubercle bacilli which are reported<sup>4</sup> to be sensitive to PAS in concentrations in the neighborhood of 0.012 mg per 100 ml of medium. These results, coupled with the effectiveness of the material in experimental tuberculosis<sup>5</sup> and the simplicity of administration, would seem to indicate that streptomycin PAS is worthy of clinical trial in human tuberculosis on an experimental basis.

**Summary.** Streptomycin PAS may be given intramuscularly without undue irritation. Chemical assay of PAS, and bioassay of streptomycin, each give virtually quantitative recovery of streptomycin PAS *in vitro*. Following intramuscular administration of streptomycin PAS in human subjects, streptomycin was demonstrated in the plasma and urine within the expected range. PAS also appeared in significant concentrations, but the quantitative relation to streptomycin was altered.

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fore, been accepted as a baseline. The addition of streptomycin PAS to water or serum gives recoveries, by the above methods, very near the expected values for both PAS and streptomycin. This would appear to indicate that the chemical union does not interfere with the biologic activity of streptomycin nor with the chemical determination of PAS.

**Results and discussion.** The plasma streptomycin concentrations, measured in the subjects who received streptomycin PAS intramuscularly, varied but were within the range expected for an equivalent amount of streptomycin. The values obtained for plasma PAS likewise varied considerably, particularly in the early minutes of absorption. Peak values developed between 15 and 30 minutes after intramuscular injection and averaged 1.35 mg per 100 ml in the subjects receiving an amount of streptomycin PAS equivalent to 1.0 g of streptomycin. In those subjects receiving a dose one-half this size the peak plasma concentrations averaged 0.6 mg per 100 ml. Concentrations fell rapidly and were at the base line after 4 hours in the group receiving the smaller dose and only very slightly above the base line in those who received the larger amount. This data is illustrated in Fig. 1. In Fig. 2, the curve of plasma PAS following ingestion of 0.8 g of PAS is compared with that obtained, in the same individual, following intramuscular administration of an equivalent amount of PAS as streptomycin PAS. The curves are very similar save that the peak concentration appeared later when the material was taken orally. Virtually the same relationships were observed in a second individual.

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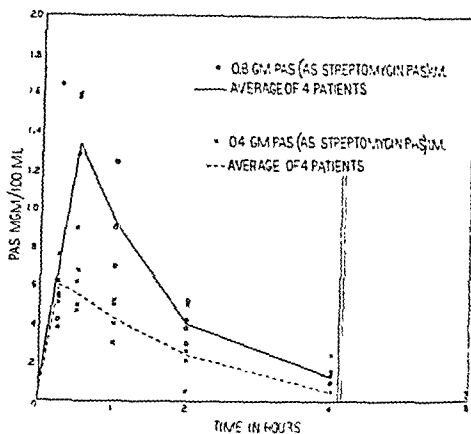


FIG. 1.  
Plasma concentrations of PAS following intramuscular administration of streptomycin PAS in amounts equivalent to 1 g of streptomycin (0.8 g PAS) and 0.5 g streptomycin (0.4 g PAS). The lines indicate average values for each dosage level.

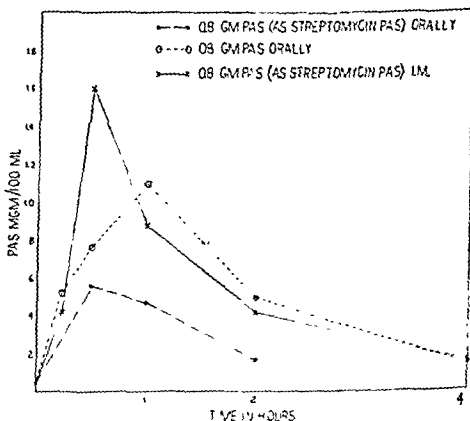


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Comparison of plasma concentrations of PAS following equivalent amounts of: (1) Streptomycin PAS intramuscularly. (2) Streptomycin PAS orally. (3) PAS orally.

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<sup>7</sup> Way, E. L., Smith, P. K., Howie, D. L., Weiss, R., and Swanson, R., *J. Pharm. and Exp. Therap.*, 1948, 93, 369.

TABLE I. Activities in  $\mu\text{g}$  Phosphorus Liberated in 15 Min. at 37.5°C per mg N.

Fraction	14 days gestation			19 days gestation			Activity of individual fraction			1-2 days after birth			9-10 days after birth		
	No. rats	Range	Avg	No. rats	Range	Avg	No. rats	Range	Avg	No. rats	Range	Avg	No. rats	Range	Avg
R	2	66-80	73	2	480-500	490	3	720-1000	850	3	610-720	670			
M <sub>1</sub>	3	55-140	91	3	550-680	615	4	720(1400)*-1700	1300(1500)	3	860-1300	1050			
M <sub>2</sub>	3	50-88	70	3	82-88	85	4	110(560)-870	530(670)	4	420-850	570			
S	4	170-306	230	2	410-420	415	4	460(630)-850	660(720)	3	720-1100	920			
W	2	120-130	120	2	400-890	645	4	830-980	910	4	800-1300	970			
					Activity calculated per mg N of whole homogenate										
R		47			200			360			300				
M <sub>1</sub>		5			54			180(190)			280				
M <sub>2</sub>		15			40			48(62)			50				
S		66			250			170(190)			230				
Σ		133			544			758(802)			850				
W		120			490			910			970				
(W)		95			570			570			950				

Σ = The sum of activities of single fractions per mg N of the whole homogenate.

(W) = Values recalculated from previous data for activities of the whole homogenate corrected for increase in activity in the presence of KCl.  
 \* The wide spread is due to one determination which is out of line with the others. If this experiment were discarded the spread would be as indicated by the figures in parentheses.

fraction (fractional activity) or per mg of N in the whole homogenate (relative activity). The first way of computing indicates the changes of activity which occur during the development within the respective fraction, independent of the changes of the quantity of this fraction. The second method of computation provides an index for the contribution of enzymatic activity of one particular fraction relative to the enzymatic activity of the whole homogenate of muscle tissue. If the enzymatic activities for the single fractions computed for the total nitrogen are added, the activity obtained theoretically should be equal to the activity of the whole unfractionated tissue homogenate. The latter data correspond to the figures represented in the graph in the previous paper.<sup>1</sup> The figures obtained by computation for the total nitrogen, (relative activities) are dependent not only upon the activity in the respective fraction but also upon the quantity of this fraction relative to the other fractions composing the total homogenate. In order to show the scattering of our results we have listed in Table I the numbers of samples used and the range for the fractional activities and also the averages for single fractions and for the whole homogenate as well as the relative activities for the single fraction and for the sum of the fractions to be compared with the activity of the whole unfractionated tissue. From these figures it is quite evident that all fractions show a marked increase in activity. It can be noticed that in the 3 fractions, R, M<sub>1</sub>, M<sub>2</sub>, almost the entire increment is found for the development from the 14th to the 19th day of gestation, the period in which the most pronounced steps of structural and functional differentiation are taking place. Fraction S increases at a steadier rate over the whole developmental period investigated. The increase in the relative activities (calculated per mg nitrogen of the total homogenate) differs from the fractional activities by its dependence upon the quantity of the respective fraction. A closer correlation of the two sets of figures was not attempted. However it can be clearly recognized that in the case of fractions R, M<sub>2</sub>, and S the increases in the relative activities are of the same order of



# Liberation of Inorganic Phosphate from Adenosinetriphosphate by Fractions Derived from Developing Rat Muscle.\* (17466)

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The systematic investigation of chemical changes occurring in differentiating muscle tissue was begun<sup>1,2</sup> by assaying quantitatively the main protein fractions at different stages of development. The direct continuation of this analysis was not immediately possible because the preparative characterization of the most significant muscle proteins, the myosin group, was impeded by the similarity in solubility of the nucleoproteins<sup>3</sup> and the discrepancy between the increase in quantity of the myosin proteins and the activity of adenosinetriphosphatase which usually are thought to be closely associated. As an explanation of this discrepancy (1) an appreciable increase in the enzymatic activity in fractions other than the myosin proteins, or (2) an increase in activity of the myosin fraction itself or (3) a simultaneous occurrence of both have been considered. These possibilities have been tested experimentally and the results are here described.

**Experimental:** ATPase activity determinations. About 500 mg of fresh, minced muscle tissue were homogenized in 10 cc of .5 M KCl in the cold. The pH was adjusted to 7.5 and the homogenate was divided into two 5 cc portions. One was stored in the cold for a determination on the whole (W). The other was subjected to protein fractionation as follows: the homogenate was stirred for one hour at 40°F, and then centrifuged for ½ hour at 3,000 r.p.m. The residue is R. The supernatant was carefully adjusted to pH 5.7 by adding split drops of .04 N HCl. The insoluble material was separated by cen-

trifugation for ½ hour and denoted as M<sub>1</sub>. The supernatant was diluted with 2 volumes of water, and then centrifuged for ½ hour. The residue is M<sub>2</sub>. The supernatant is S. The fractions so separated were rehomogenized in 5 cc of .5 M KCl, and the pH was adjusted to 7.2-7.5. After dilution of each fraction and the whole, so that the activity would fall within a measurable range, .2 cc portions were taken for ATPase activity determinations, which were performed by the procedure of Dubois and Potter.<sup>4</sup> The ATP was prepared according to the method of Dounce, *et al.*<sup>5</sup>

Tubes set up for phosphate determination contained the following: .60 cc of 2.5% ammonium molybdate in 5 N H<sub>2</sub>SO<sub>4</sub>, .25 cc of sample, distilled water to dilute to 3.00 cc, and lastly, 15 cc of .25% 1-amino, 2-naphthol, 4-sulfonic acid. Phosphate standards and a blank were run concomitantly. The contents were thoroughly mixed and heated in a water bath at 37°C for 5 minutes. After cooling the samples to room temperature, colorimetric readings were taken on the Beckman spectrophotometer at 660 mμ.

Nitrogen determinations for each fraction in suspension were made by the Kjeldahl method modified by Boell<sup>6</sup> after precipitation with trichloroacetic acid (100 g to 100 cc) to yield a solution concentration of 5%.

**Results and discussion:** The enzymatic liberation of inorganic phosphate from adenosine triphosphate by 4 protein fractions obtained from mesoderm or muscle tissue at different stages of development is shown in Table I. The activities are computed as phosphate liberated *either* per mg of N in the

\* Supported in part by a grant from the American Cancer Society through the NRC Committee on Growth.

1 Herrmann, Heinz, and Nicholas, J. S., *J. Exp. Zool.*, 1948, **107**, 177.

2 Herrmann, Heinz, and Nicholas, J. S., *J. Exp. Zool.*, 1948, **107**, 165.

3 Herrmann, Heinz, and Nicholas, J. S., *J. Exp. Zool.*, 1949, **112**, 253.

4 Dubois, K. P., and Potter, V. R., *J. Biol. Chem.*, 1943, **150**, 185.

5 Dounce, A. L., Rothstein, A., Beyer, G., Thannhauser, Meier, R., and Freer, R. M., *J. Biol. Chem.*, 1948, **174**, 361.

6 Boell, E. J., *Trans. Conn. Acad. Arts and Sci.*, 1945, **36**, 429.

oping muscle obtained from rat embryos of 14 days, fetuses of 19 days, rats of 1-2 days and 9-10 days of age. The activity was calculated in micrograms of phosphorus liberated in 15 min. at 37.5° per mg N.

The activity was determined for each of 4 fractions and the sum of these, the activity of the whole homogenate, was in close agreement with previous determinations of the whole homogenates.

All 4 fractions increase in activity during development; the  $M_1$  fraction, containing the purest preparation of the myosin proteins develops in activity 6 times faster than the other fractions.

While the apyrase activity was formerly thought to be limited to the myosin protein fraction this contributes but a minor part of the total activity.

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## Role of Pyridoxine in the Production of Leucocytes in Normal and Leukemic Mice.\* (17467)

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Morgan, Groody, and Axelrod<sup>1</sup> showed that pyridoxine-deficient dogs developed a leucocytosis with an absolute increase in the numbers of circulating neutrophils and an absolute decrease in the numbers of lymphocytes. These abnormalities could be corrected by giving adequate doses of pyridoxine. In the same year McCall, Waisman, Elvehjem, and Jones<sup>2</sup> described similar leucocyte changes in pyridoxine-deficient and in pantothenic acid-deficient monkeys. They stated that the reversal of the polymorphonuclear to lymphocyte ratio also occurred in riboflavin deficiency. In the pyridoxine-deficient animals the reversal could not be corrected completely by pyridoxine but could be corrected by whole liver. Wintrobe and his associates<sup>3</sup> observed a tendency of the granulocytes to increase in pyridoxine-deficient swine, but there was no reversal of the

ratio of polymorphonuclears to lymphocytes. Stoerk, Eisen, and John<sup>4</sup> described thymus atrophy and a reduction in the number of fixed and circulating lymphocytes, and Stoerk<sup>5</sup> described lymphoid atrophy and regression of rat lymphosarcoma in pyridoxine-deficient rats. In order to facilitate the production of pyridoxine deficiency, Ott<sup>6</sup> investigated the antipyridoxine activity of desoxypyridoxine hydrochloride (2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine). He found that two molecules of the analogue were sufficient to offset the vitamin activity of one molecule of pyridoxine, as measured by chick growth. Under other conditions<sup>7</sup> much larger quantities of the analogue are necessary. These observations prompted us to investigate the effect of both pyridoxine deficiency and pyridoxine excess on the circulating leucocytes and hematopoietic organs of normal mice and of mice having various abnormalities of the leucocyte pattern.

### *Pyridoxine Deficiency in Normal Mice.*

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\* This research was made possible by grants from the National Vitamin Foundation and the Price McKinney Memorial Fund.

<sup>1</sup> Morgan, A. F., Groody, M., and Axelrod, H. E., *Am. J. Physiol.*, 1946, **146**, 723.

<sup>2</sup> McCall, K. B., Waisman, H. A., Elvehjem, C. A., and Jones, E. S., *J. Nutrition*, 1946, **31**, 685.

<sup>3</sup> Wintrobe, M. W., Follis, R. H., Miller, M. H., Stein, H. J., Alenayaga, R., Humphreys, S., Suksta, A., and Cartwright, G. E., *Johns Hopkins Hosp. Bull.*, 1943, **72**, 1.

<sup>4</sup> Stoerk, H. C., Eisen, H. N., and John, H. M., *J. Exp. Med.*, 1947, **85**, 365.

<sup>5</sup> Stoerk, H. C., *Fed. Proc.*, 1948, **7**, 281.

<sup>6</sup> Ott, W. H., *Proc. Soc. Exp. Biol. and Med.*, 1946, **61**, 125.

<sup>7</sup> Umbreit, W. W., and Waddell, J. G., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 293.

magnitude as the increases in fractional activities. In contrast to these fractions an exceptionally great increase in the relative activity is found in the  $M_1$  fraction. In this instance an increase of 14 times for the fractional activity is to be compared with an increase of about 56 times for the relative activity. This difference is due to the rapid increase in the quantity of the  $M_1$  fraction which has been described before.

The values for the relative activities in Table I, when added ( $R$ ,  $M_1$ ,  $M_2$ ,  $S$ ) should equal the activities found in the whole homogenate ( $W$ ). Since the two sets of figures are in close agreement, it can be concluded that the values secured for the relative activities give a correct measure for the proportion in which these individual fractions contribute to the activity of the whole homogenate. The increase in activity for the whole homogenate found in this series corresponds closely to the values obtained previously.<sup>3</sup> The present values are consistently somewhat higher than the previous ones since the activity of the whole when homogenized in KCl is 18% higher than when homogenized in water, due to activation by the K ion. For the first day after birth the earlier value ( $W$ ), which is more than 35% lower than the recent value  $W$ , forms a "dip" in the curve in previous experiments. Since the rate of increase in activity is greatest up to the time of birth, the present higher value,  $W$ , is the more reasonable one. The difference between the rapid increase in enzymatic activity and the slow increase in the quantity of the total fraction of myosin proteins (the sum of the present fractions  $M_1 + M_2$ ) which appeared previously as a discrepancy can now be explained. The former assumption was that most of the apyrase activity in muscle at all stages of development should be linked to the myosin protein fraction and should remain fairly constant for the unit of this fraction. It now appears that only a minor part of the total activity is contributed by the myosin proteins; although a large increase in activity does occur within the myosin fraction itself.

The problem of the nature of the proteins of the myosin group as well as the relationship

of the different enzymes with apyrase activity is at present in a state of flux. As to the former, discussion centers around Szent-Gyorgyi's<sup>7</sup> separation of the myosin complex into actin and myosin, and recently Bailey seems to have found evidence for a new member of the myosin family (his tropomyosin). Enzymes with apyrase activity and distinctly different properties recently have been described from various sources<sup>8-10</sup> and even in muscle a distinction of ATPase enzymes was recently claimed. The fact that there is an increase during embryonic differentiation of all fractions of muscle tissue is in agreement with the observations of Potter,<sup>11</sup> Flexner<sup>12</sup> Barth,<sup>13,14</sup> and Moog<sup>15</sup> who find an increase in apyrase enzymes during differentiation in a great variety of mammalian and even invertebrate tissues. In these instances the increase means a more or less indirect role of apyrases in the elaboration and maintenance of specific structures and functions. In the  $M_1$  fraction this relation is a more direct one since the development of enzymatic and structural properties of the myosin proteins in muscle represents directly the products of differentiation in a highly specialized tissue.

**Summary.** In a quantitative chemical study of developing muscle there is a greater activity of adenosinetriphosphatase than is warranted by the increase in amount of myosin proteins. In order to ascertain the reason for this discrepancy determinations of phosphatase activity were made on fractions of devel-

<sup>7</sup> Szent-Gyorgyi, A., *Chemistry of Muscular Contraction*, Academic Press, Inc., New York, 1947, 150 pp.

<sup>8</sup> Barth, L. G., and Jaeger, L., *J. Cell. and Comp. Physiol.*, 1947, **30**, 111.

<sup>9</sup> Kielley, W. W., and Meyerhof, O., *J. Biol. Chem.*, 1948, **174**, 387.

<sup>10</sup> Zeller, F. A., *Experientia*, 1948, **15**, 194.

<sup>11</sup> Potter, V. R., Schneider, W. C., and Liebl, G. J., *Cancer Research*, 1945, **5**, 21.

<sup>12</sup> Flexner, J. B., and Flexner, L. B., *J. Cell. and Comp. Physiol.*, 1948, **31**, 311.

<sup>13</sup> Barth, L. G., *J. Trans. N. Y. Acad. Sci.*, 1919, Ser. II, Vol. II, 108.

<sup>14</sup> Jaeger, L., and Barth, L. G., *J. Cell. and Comp. Physiol.*, 1948, **32**, 319.

<sup>15</sup> Moog, F., *J. Exp. Zool.*, 1947, **105**, 209.

leucocyte pattern to normal. Total and differential leucocyte counts were done twice weekly. At autopsy, sections of liver, spleen, marrow, thymus, lymph nodes, and both adrenals were taken for histologic study.

As shown in Fig. 1, a complete reversal of the ratio of granulocytes to leucocytes occurred very promptly after desoxypyridoxine was started, with development of absolute granulocytosis and lymphopenia. Within 3 days after the pyridoxine supplement was started restoration of the normal leucocyte pattern had begun, and was complete 14 days later (36th day). After the reduction of the dose of desoxypyridoxine during the initial period, only one animal died and the others remained in apparent good health. There was no significant weight loss, and they did not develop ruffled fur, convulsions, or obvious neurologic disturbances. Red blood cell counts were not done in this experiment, but, in a preliminary experiment which was conducted under identical conditions except for a lower dose of supplementary pyridoxine, the mean erythrocyte count did not change significantly.

At autopsy on the 36th day no gross or microscopic abnormality could be detected in the tissues. In order to obtain material for histologic study at a time when the reversal of the granulocyte to lymphocyte ratio was established, 20 mice were maintained on the regimen described above, but were not given the supplementary pyridoxine. Fifteen of the 20 survived to be killed on the 37th day, at which time granulocytosis and lymphopenia, with a complete reversal of the ratio, had been present for 32 days. None of these animals showed any evidence of lymphoid atrophy. The thymus glands and lymph nodes were normal in appearance, and the splenic follicles were, if anything, slightly hyperactive. There was no increase in the size of the adrenal glands or demonstrable change in their cytological structure. The marrows showed possible increase in cellularity due to increased numbers of granulocytes without increase in immature cells, although this change was not marked. The liver of one animal that died early showed extensive hemorrhagic necrosis. The liver of another that survived to be killed showed a few small foci of necrosis. There is reason to

believe that a generalized tissue deficiency of pyridoxine had not been established. Growth was not impaired, convulsions did not occur, there was very slight anemia, and lymphoid regression or atrophy was not present at autopsy. It is likely that a partial deficiency is sufficient to produce the leucocyte shift without producing other demonstrable changes.

*Effect of Pyridoxine Deficiency on the Leukemia of Ak Mice.* In mice of the Ak strain the intravenous injection of cells from diseased older animals produces leukemia in 100% of younger animals.<sup>9</sup> In the animals now being bred in this laboratory this is believed to be a mixed type of leukemia, in which both adult granulocytes and adult lymphocytes participate, with the appearance of small numbers of immature blastic cells. About 10 to 15 days after transplanting, the total leucocyte count begins to rise. It remains elevated for 10 to 20 days and then falls toward normal levels shortly before the animal dies. Death usually occurs 25 to 35 days after the transplant. Differential counts show that the initial rise is due to an increase of granulocytes. The ratio of these cells to lymphocytes may be as great as 4 to 1. As the leukemia progresses the lymphocytes increase and, coincidentally with this rise, the granulocytes begin to fall and reach normal levels when the lymphocytes are still elevated. At death any elevation of counts above normal is due to increased numbers of lymphocytes. The immature blastic cells appear in small numbers at the height of the leucocytosis and persist until death. The effect of pyridoxine deficiency on this leucocyte pattern was investigated. Fourteen weanling Ak mice were placed on a diet of Purina dog chow. On the first day all animals were given intravenous injections of a suspension of cells from lymph node and spleen of a diseased older animal. On the 8th day a group of 9 was started on desoxypyridoxine hydrochloride. Each was given 1.5 mg subcutaneously twice daily. Total and differential leucocyte counts were done at the end of the first week and twice weekly

<sup>9</sup> Weir, D. R., and Heinle, R. W., *Proc. Soc. Exp. Biol. and Med.*, 1947, **66**, 268.

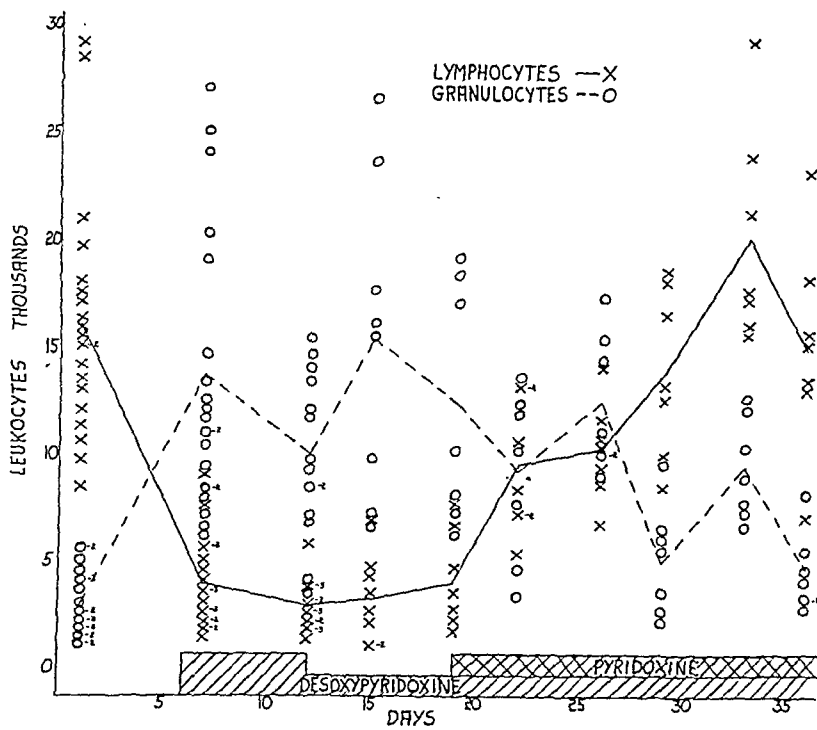


Fig. 1.  
Leucocyte changes in pyridoxine-deficient normal mice.

Weanling mice of a Swiss strain were placed in cages with wire-mesh bottoms. They were fed *ad libitum* a modification of a pteroylglutamic acid-deficient diet which has been described previously;<sup>8</sup> pyridoxine and succinylsulfathiazole were not included. The animals were given pteroylglutamic acid intraperitoneally in daily doses of 20  $\mu$ g. In addition desoxypyridoxine<sup>†</sup> was given subcutaneously as a 2% solution of the hydrochloride. In a preliminary experiment, daily doses in excess of 1 mg were fatal within ten days. On a 1 mg dose the mean survival time was 20 days and weight loss was marked. Many of the animals developed the expected granulocytosis and lymphopenia but several did not. In a second experiment the animals were given 2 mg daily for a week and then 1 mg daily. Again the leucocyte response was variable and weight loss occurred rapidly. Also the leucocyte changes could not be corrected completely

by the administration of pyridoxine. These unsatisfactory results using a purified diet suggested a trial of a crude diet (Purina dog chow) supplemented by larger amounts of desoxypyridoxine given parenterally, as a possible means of producing the desired alteration in the leucocyte pattern. This approach proved successful. Twenty Swiss mice were fed Purina dog chow *ad libitum*. On the 6th day subcutaneous injections of desoxypyridoxine HCl in a 2% solution were started. From the 6th to the 12th day 8 mg were given daily in 2 doses of 4 mg each at an interval of 8 hours. Because of a high mortality in the animals the doses were then halved to supply 4 mg daily, and this was continued to the end of the experiment; during this period only one mouse died. Supplementary pyridoxine HCl (0.2%) was added to the ground diet on the 19th day. Since the mice ate from 3 to 6 g of dog chow daily, they were supplied with 6 to 12 mg of supplementary vitamin daily. Smaller amounts used in preliminary experiments had failed to restore the

<sup>8</sup> Weir, D. R., Heinle, R. W., and Welch, A. D., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 211.

<sup>†</sup> Kindly furnished by Merek & Co., Inc.

transaminase and co-decarboxylase.<sup>15-18</sup> In the intact normal animal the various compounds of the group presumably are freely interconvertible.<sup>19,20</sup> From these considerations, the hypothesis is suggested that the granulocytosis in pyridoxine-deficient mice is due to the failure on the part of the animals to transaminate, decarboxylate, or otherwise modify unneeded supplies of granulocyte-building substances, and that the animals thereupon utilize these unneeded supplies in producing excess numbers of granulocytes. It is not necessary to assume that the reverse would be true. A further hypothesis may be made that the granulocytosis of a disease state such as human myeloid leukemia may be due to a defect in the absorption or utilization of vitamins of the B<sub>6</sub> group. Although freely interconvertible in the intact animal, this may not be true of them in the diseased animal.

<sup>15</sup> Gunsalus, I. C., Bellamy, W. D., and Umbreit, W. W., *J. Biol. Chem.*, 1944, **155**, 685.

<sup>16</sup> Lichstein, H. C., Gunsalus, I. C., and Umbreit, W. W., *J. Biol. Chem.*, 1945, **161**, 311.

<sup>17</sup> Ames, S. R., Sarma, P. S., and Elvehjem, C. A., *J. Biol. Chem.*, 1947, **167**, 135.

<sup>18</sup> Beiler, J. M., and Martin, G. J., *J. Biol. Chem.*, 1947, **169**, 345.

<sup>19</sup> Sarma, P. S., Snell, E. E., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 284.

<sup>20</sup> Junqueira, P. B., and Schweigert, B. S., *J. Biol. Chem.*, 1948, **174**, 605.

Further investigations in mice and in leukemic human beings are in progress to test the validity of these hypotheses. It has been demonstrated that adrenocorticotrophic and adrenal cortical hormones will cause granulocytosis and lymphopenia in animals.<sup>21,22</sup> In our histologic material we could not demonstrate evidence of adrenal cortical hyperplasia.

**Summary.** 1) Pyridoxine deficiency in mice produces granulocytosis and lymphopenia. 2). The leukemia of the Ak mice now being bred in this laboratory is participated in by both granulocytes and lymphocytes. In pyridoxine deficiency the leukemic granulocytosis is more severe and the survival time of the animals is decreased. 3) The hypothesis is advanced that the granulocytosis in pyridoxine-deficient mice is due to the failure on the part of the animals to perform metabolic functions in which pyridoxine derivatives play a catalytic role; thus, supplies of compounds used in the formation of granulocytes accumulate, and, as a result, the animals utilize this plethora in producing excess numbers of granulocytes. The implications of this hypothesis are discussed.

<sup>21</sup> Valentine, W. N., Craddock, C. G., Jr., and Lawrence, J. S., *Blood*, 1948, **3**, 729.

<sup>22</sup> Hills, A. G., Forsham, P. H., and Finch, C. A., *Blood*, 1948, **3**, 755.

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## Device for Measuring Blood Pressure in the Unanesthetized Rat. (17468)

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The simple technic for measuring blood pressure in the rat described here consists of placing a cuff permanently around the abdominal aorta and vena cava just above their bifurcations and measuring the blood pressure by means of an oscillometer and mercury manometer.

### *Description of the Apparatus and Its Use.*

The aortic cuff is shown in Fig. 1. It consists of a latex bulb with a short stem which

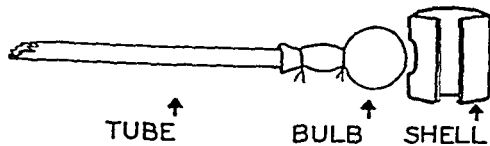


Fig. 1.  
The aortic cuff unassembled.

is fitted on to a piece of plastic tubing, and a plastic shell into which the bulb is inserted.

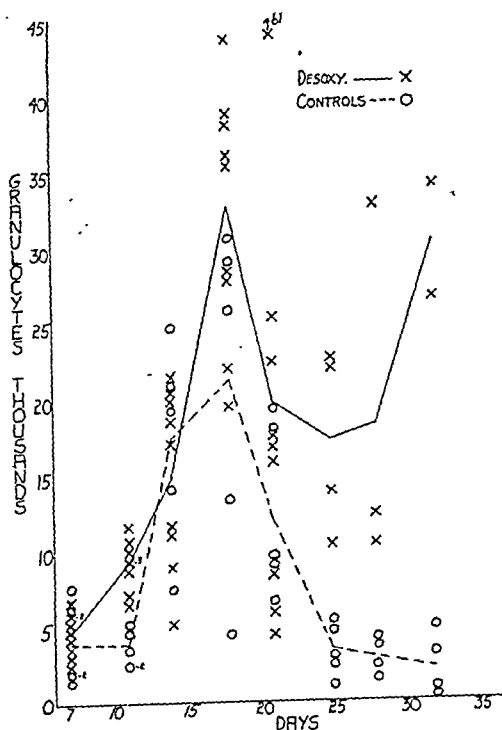


FIG. 2.

The effect of pyridoxine deficiency on the granulocytosis of Ak mouse leukemia.

thereafter. The mean survival time of the animals receiving desoxypyridoxine was 24.6 days as compared to 31.8 days for the controls. This decrease in survival time is statistically significant. The value for *P* is less than 0.02.<sup>10</sup> The leucocyte patterns are shown in Fig. 2. In the early stages of the leukemia the granulocyte counts of the experimental animals were generally higher than those of the controls. On the 25th day and thereafter all the granulocyte counts of the experimental animals were 2 to 6 times as great as, the highest count shown by the controls during the same period. The lymphocyte counts of the experimental animals were generally lower than those of the controls but not significantly so. Histologic study of sections of liver, spleen, bone marrow and lymph nodes taken at autopsy failed to reveal any difference between the experimental and control groups.

<sup>10</sup> Fischer, R. A., *Statistical Methods for Research Workers*, Oliver and Boyd, Edinburgh, 1934.

*Preliminary Studies of the Effects of Pyridoxine in Excess.* Groups of normal mice were given parenteral pyridoxine HCl in divided daily doses of 8 and 16 mg for a period of 48 days. None of the animals showed any significant change in the leucocyte pattern. Three groups of 5 Ak mice were injected intravenously with a leukemic transplant. All were maintained on dog chow diet. One group received 2 mg of parenteral pyridoxine HCl in a single daily dose; another group received 4 mg; and the third group served as controls. The pyridoxine supplement failed to influence the course of the leukemia or to alter the histologic findings at autopsy. During the study of a myeloid metaplasia factor from human urine Heinle, Hirschmann, and Wearn<sup>11</sup> observed that the injection of certain extracts of urine from patients with chronic myeloid leukemia, produced a moderate to marked granulocytosis in mice. The effects of excess amounts of pyridoxine on this granulocytosis was studied. Ten adult Swiss mice were given for 10 days daily injections of a urine extract known to be potent. Five of these also were given pyridoxine hydrochloride parenterally in doses of 8 mg twice daily. The mean polymorphonuclear count of the 10 animals at the beginning of the experiment was 3370 per cu mm. At the end of the 10 day injection period the mean for the group receiving pyridoxine was 32,615; for those not receiving pyridoxine it was 43,544. The difference is not statistically significant.

*Discussion.* The results indicate that a deficiency of pyridoxine causes an increase in granulocyte production in normal mice and in mice dying from transplanted leukemia. It is known that vitamins of the B<sub>6</sub> group take part in transamination and decarboxylation,<sup>12-14</sup> and that the active compound is a phosphorylated form of pyridoxal acting as co-

<sup>11</sup> Heinle, R. W., Hirschmann, H., and Wearn, J. T., *Approaches to Tumor Chemotherapy*, 1947, 77.

<sup>12</sup> Schlenk, F., and Snell, E. E., *J. Biol. Chem.*, 1945, **157**, 425.

<sup>13</sup> Bellamy, W. D., and Gunsalus, I. C., *J. Bact.*, 1943, **46**, 573.

<sup>14</sup> Gunsalus, I. C., and Bellamy, W. D., *J. Bact.*, 1944, **47**, 413.

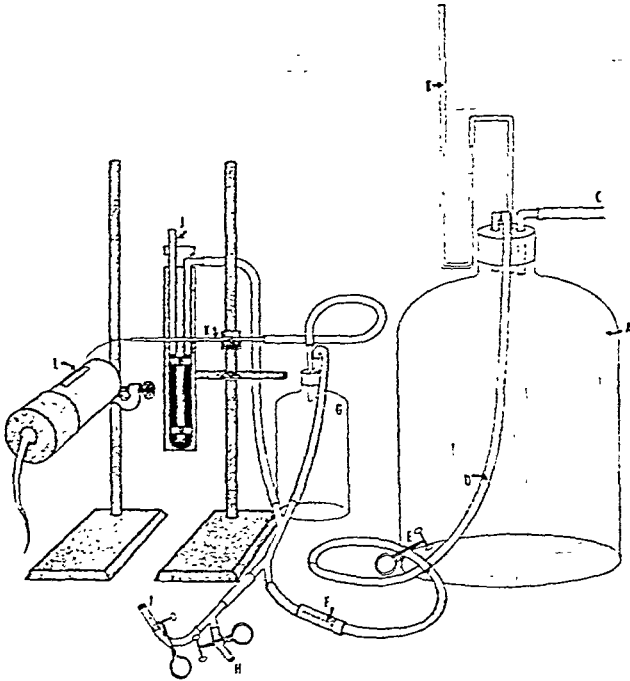


FIG. 3.

Oscillometer and manometer hooked up with pressure bottles. The oscillometer is connected to the tubing from the animal.

aorta and vena cava a few days before the first readings were made. Afterwards the blood pressure measurements were determined 3 times a week or oftener, over a period of 8 weeks. These readings are shown in Table I. The average of 540 blood pressure readings taken on the 20 normal unanesthetized rats over a 2 month period was 130 mm Hg.

The readings obtained by the cuff method were compared with the systolic pressure in the femoral artery measured intra-arterially by means of a capacitance manometer designed by Noble.<sup>1</sup> These readings were made on rats anesthetized with ether. Immediately after the intra-arterial reading, while the animal was still asleep, blood pressure readings by the cuff method were made. Twenty-nine rats were used for this study. Ten of this group had been made hypertensive by enclosing the left kidney with a latex capsule and removing the right kidney (Abrams and So-

bin<sup>2</sup>). The remaining 19 rats were the normal animals which had been used previously for the blood pressure readings recorded in Table I. The comparative data are presented in graph form in Fig. 4. The intra-arterial systolic pressure for a given rat is plotted as the ordinate and the pressure reading by the cuff method as the abscissa. The diagonal line represents perfect agreement. The readings by the 2 methods in both normal and hypertensive animals tend to fall along this line. The average intra-arterial systolic pressure for the 19 normal animals was  $121 \pm 15$  mm Hg and the reading by the cuff method while the animals were still under ether was  $118 \pm 15$  mm Hg. Because of the close correlation of the readings by the 2 methods it is assumed that the readings by the cuff method indicate the approximate systolic blood pressures. It has been noted, however, that when the cuff is inflated to the "systolic pressure" reading the aorta may not be com-

<sup>1</sup> Noble, F. W., Third Conference on Factors Regulating Blood Pressure, May 5-6, 1949, Josiah Macy, Jr. Foundation, New York.

<sup>2</sup> Abrams, M., and Sobin, S., *Proc. Soc. Exp. Biol. and Med.*, 1947, 64, 412.



To make the bulb a glass bead 4 mm in diameter with a stem attached is dipped into liquid latex. After drying for 24 hours, the latex is vulcanized by exposure to sulfur chloride fumes for one minute, and then washed under running tap water. After drying, the bulb is powdered and peeled off the mold. The short stem of the bulb is fitted into the end of a 12 cm piece of 18-gauge polyethylene tubing. The tubing is reinforced where it is fitted into the capsule by a 4 mm length of 18-gauge stainless steel tubing. The shell of the cuff consists of a plastic tube (Tenite II) 6 mm long with an inner diameter of 4 mm and an outer diameter of 6 mm. The shell is split lengthwise so that it can be slipped around the aorta. A 3 mm hole is drilled in the shell just opposite the slit and midway between the two ends. The bulb is fitted into the shell, with the tubing brought out through the hole. The bulb should just fill the inside of the shell. The cuff is washed in hot running tap water for 4 hours and then stored in 70% alcohol. Before use, each cuff is tested to withstand an air pressure of 300 mm Hg.

To place the cuff on the aorta the animal is anesthetized with ether and a midline incision is made in the abdomen. The aorta and vena cava are dissected free from the surrounding tissue, but are not separated from each other. Isolating the aorta was found to be difficult because of the danger of tearing the vena cava in the operation. The cuff is placed around both vessels just above their bifurcations. The shell is tightly closed with a strong ligature and secured firmly to the surrounding tissues. The tubing is brought through the posterior wall of the abdominal cavity and carried under the skin to the back of the neck where it is brought to the outside. In this position the tube cannot be disturbed by the animal (Fig. 2). Special care must be taken to prevent moisture from accumulating in the tube or bulb which could falsify pressure readings. There is no evidence that the animal suffers any inconvenience from the cuff. Autopsy examination of the aorta with the cuff *in situ* made 2 to 3 months after inserting the cuff has revealed very little fibrous tissue formation.

Blood pressure readings are made with the



Fig. 2.  
Rat showing place of exit for the plastic tubing leading from cuff.

use of an improvised oscillometer and a mercury manometer. This apparatus is shown in Fig. 3. The animal is placed in a metal holder (L) or in a 500 cc beaker. There is no struggling since the animal is not uncomfortable or irritated in any way. The tube from the cuff is connected with an oscillometer which consists of a 15 mm length of 1 mm capillary tubing (K) connected with a pressure bottle (G). The capillary tube contains a small drop of colored alcohol which serves as an indicator of the oscillations which are transmitted from the aorta. The pressure bottle G has a mercury manometer attached and it in turn is connected with a bottle of compressed air (A). The cuff is inflated through the capillary oscillometer with air supplied at a steady smooth flow through a hypodermic needle (F) from the reservoir A which has been adjusted to deliver pressures from 0 to 300 mm Hg in about 30 seconds. As the pressure rises in the cuff and oscillometer, the alcohol bead travels toward the cuff and shows distinct oscillations which reach a maximum intensity, gradually decrease, then abruptly diminish. The point of diminution is taken as the endpoint. A manometer reading is made at this point. A small pulsation persists after the endpoint. The pressure in the cuff is quickly released through the side tube H. Three to 5 readings are made at one-minute intervals.

*Experimental data.* This method for measuring blood pressure in the unanesthetized rat has been tested on a group of 20 normal male rats of the Sprague-Dawley strain which weighed from 105 to 165 g at the beginning of the study. Cuffs were placed around the

## Precipitation of Insulin by Lysozyme and Effect of the Complex on the Blood Sugar Level. (17469)

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Because of the possible therapeutic application of a lysozyme-insulin complex, the properties of which might be expected to resemble those of protamine-insulin, experiments have been done on the precipitation of insulin by crystalline egg lysozyme (Armour) isoelectric point 10.5 to 11<sup>1,2</sup> and lysozyme which was treated in several different ways intended to destroy its lytic power as tested on *M. lysodeikticus*. The effect of the resuspended precipitates on the blood-sugar level of rabbits was also tested.

Table I shows the data obtained in measuring the precipitation of zinc-containing crystalline insulin dissolved in a minimal volume of N/100 HCl, adjusted to pH 7.3 with N/100 NaOH and made up to 0.10% concentration. The indicated quantities of a 0.10% (uncorrected for 9.56% moisture) solution of lysozyme were added to 1 ml of the insulin solution and the volume made up to 2 ml with water. After standing over night the tubes were centrifuged and the supernatant fluids tested for insulin and lysozyme by adding more of one or the other reagent. The precipitates were washed twice with 2 ml of water, centrifuging both times, and analyzed for nitrogen by the Kjeldahl method using H<sub>2</sub>SO<sub>4</sub> and H<sub>2</sub>O<sub>2</sub> for digestion, the color being developed with Nessler's reagent and measured in a photoelectric colorimeter. All the mixtures were set up and analyzed in duplicate.

While 0.60 ml of a 0.10% solution of untreated lysozyme precipitated almost all the insulin in 1 ml of the insulin solution,\* 96% of the total nitrogen present in the 2 reactants being found in the precipitate, 0.90 ml of lysozyme solution which had been a) dissolved in

75% ethanol and heated in a boiling water bath for 2 hours,<sup>†</sup> b) dissolved in water and heated in the autoclave at 123°C for 1/2 hour,<sup>‡</sup> c) dissolved in water and photodynamically treated (1-250,000 methylene blue and exposure of a thin layer of solution in a Petri dish to daylight for 6 days<sup>§</sup>) precipitated respectively 64%, 80% and 75% of the insulin. Untreated lysozyme added in excess of that required to precipitate all the insulin (at least up to the addition of an excess of 50%) did not change the quantity of nitrogen in the precipitate, but remained in the supernatant fluid. It was also found (not recorded in the table) that the addition of twice as much insulin as was required to combine with a given quantity of lysozyme resulted in the excess remaining in solution.

Rabbits weighing 2.5 to 2.8 kg were injected subcutaneously with 0.086 ml of lysozyme-insulin suspensions containing 20 units of insulin per ml and prepared with (a) untreated lysozyme, (b) untreated lysozyme with added zinc, (c) lysozyme in 75% ethanol solution and heated as described, (d) lysozyme in water solution and autoclaved as described, (e) lysozyme photodynamically treated as described, followed by additional exposure for 18 hours to a 200 watt Mazda lamp at a dis-

\* Taking the molecular weight of lysozyme as 13,900<sup>3</sup> and that of insulin as 32,000<sup>4</sup> (as found in 0.25% solution) it appears that 5 molecules of the former combine with 4 of insulin. Moisture content of the insulin was disregarded. The small quantity of zinc in the insulin (ca. 0.28  $\gamma$  per unit) may have an influence on this ratio.

<sup>3</sup> Fromageot, C., and Privat de Garilhe, M., *Biochim. et Biophys. Acta*, 1949, **3**, 82; Palmer, K. J., Ballantyne, M., and Galvin, J. A., *J.A.C.S.*, 1948, **70**, 906.

<sup>4</sup> Gutfreund, H., *Biochem. J.*, 1948, **42**, 156, 544.

<sup>†</sup> Lytic power for *M. lysodeikticus* was destroyed by this treatment.

<sup>‡</sup> Lytic power for *M. lysodeikticus* was about 10% of the original value after this treatment.

<sup>1</sup> Meyer, K., Thompson, R., Palmer, J. W., and Khorazo, D., *J.B.C.*, 1936, **113**, 303.

<sup>2</sup> Alderton, G., and Ferold, H. L., *J.B.C.*, 1945, **157**, 43; *ibid.*, 1946, **164**, 1.

TABLE I.

Systolic Blood Pressures as Determined by the Oscillometric Method on a Group of 20 Adult Male Rats Over a 2-Month Period.

Rat No.	Blood pressure, mm Hg weekly avg								Avg for 8-wk period
	1st	2nd	3rd	4th	5th	6th	7th	8th	
760	123	129	119	119	117	120	122	121	121
761	125	123	115	120	116	124	124	132	122
762	137	121	124	123	137	137	127	142	131
763	139	134	134	143	136	126	120	133	133
764	140	119	123	128	117	129	129	128	127
765	134	126	133	123	117	131	142	138	131
767	139	126	124	126	111	124	121	126	125
768	128	110	123	129	120	134	141	143	129
769	138	108	132	118	119	130	148	163	134
770	136	129	125	126	113	133	131	133	128
771	141	118	133	127	133	141	135	135	133
772	126	126	115	126	130	130	135	135	128
773	124	116	130	120	120	129	130	133	125
774	121	124	125	137	127	133	132	137	130
775	—	142	130	122	146	134	149	138	137
776	136	133	140	145	137	155	120	—	138
777	120	129	130	131	129	135	—	—	129
778	131	141	145	160	138	—	—	—	143
779	—	124	128	129	141	125	136	133	131
781	—	133	126	113	118	120	122	123	120
Weekly group avg	131	126	128	128	126	131	131	135	

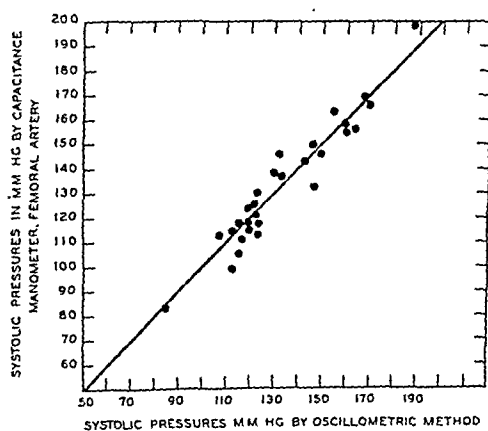


FIG. 4.

Comparison of the intra-arterial pressure by the capacitance manometer with the indirect pressure by the oscillometric method.

pletely occluded, for pulsations are still recorded by the capacitance manometer.

Simultaneous occlusion of the thoracic aorta and inferior vena cava has been shown to increase blood pressure in the dog (Barcroft<sup>3</sup>). However, in our observations no measurable change occurred in the intracarotid pressure when taken simultaneously with the pressure readings made with the cuff on the abdominal aorta and vena cava.

*Summary.* A device for measuring blood pressure in the aorta of the unanesthetized rat is described. It is simple and permits an unlimited number of readings over a long observation period.

<sup>3</sup> Barcroft, H., *J. Physiol.*, 1931, **71**, 280.

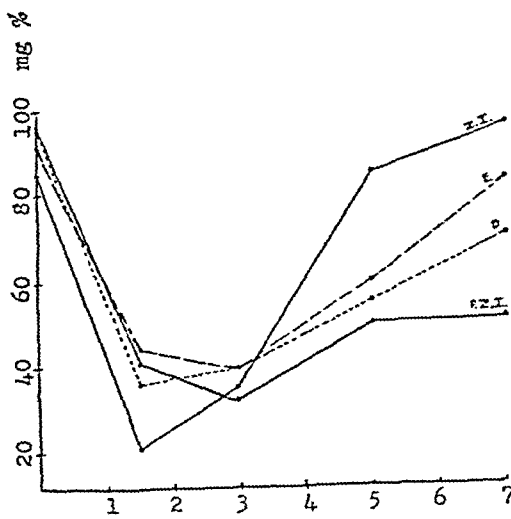
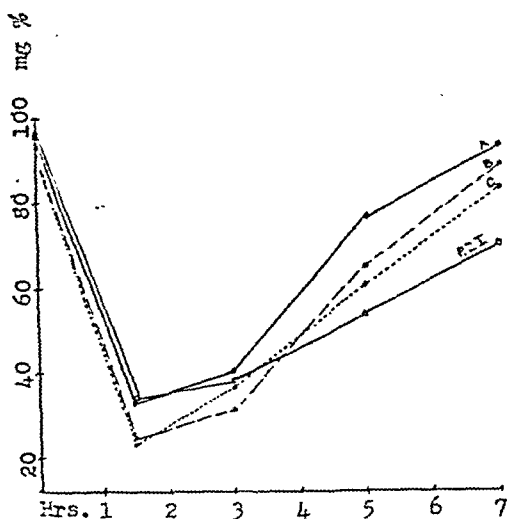


FIG. 1.

Blood-sugar curves following injection of lysozyme-zinc-insulin, protamine-zinc-insulin and zinc-insulin into rabbits. See text for description of the preparations injected.

tance of 8 inches.<sup>§</sup> Three  $\gamma$  of zinc<sup>||</sup> per unit of insulin were added to samples B, C, D and E.

Samples A, B and C were tested simultaneously, along with a protamine-zinc-insulin reference standard containing 40 units of U.S.P. zinc-insulin and 91.5  $\gamma$  of zinc per ml.,<sup>\*</sup> of which 0.043 ml were injected. On the second day, the remaining 2 samples of lysozyme-insulin were tested, along with the reference standard of protamine-zinc-insulin and a solution of U.S.P. zinc-insulin crystals containing 40 units per ml, of which 0.043 ml were administered. Blood was drawn from the marginal ear vein before, and 1½, 3, 5 and 7 hours after administering each of the insulin preparations to 6 rabbits, and was analyzed by the U.S.P. XIII method.

The average blood-sugar values found in the fasting state and 1½, 3, 5 and 7 hours after the injections for the 6 animals\*\* in each group are shown graphically in Fig. 1.

<sup>§</sup> Lytic power for *M. lysodeikticus* was about 1% of the original value after this treatment.

This, and the use of 50% more lysozyme than was required to precipitate all the insulin when using untreated lysozyme, resulted in complete precipitation of insulin and the presence of lysozyme in the supernatant fluid of all the mixtures.

<sup>\*</sup> This is somewhat less than the 3  $\gamma$  zinc per unit of insulin in samples B, C, D, and E.

In both days' experiments the values, 1½ hours after administering lysozyme-insulin, were either approximately the same, or somewhat lower than following protamine-zinc-insulin. Between 1½ and 3 hours, 4 of the 5 curves for lysozyme-insulin and 1 for protamine-zinc-insulin were slowly rising. Five hours after the injections the blood-sugar values were, respectively, 80%, 70%, 66%, 58% and 66% of the fasting levels for samples A, B, C, D and E and 56% and 52% for the 2 protamine-zinc-insulin samples. Two hours later the corresponding figures were 98, 92, 89, 74 and 91 and 73 and 53. In the case of insulin in solution the blood-sugar was back to the fasting level in 5 hours.

If any therapeutic application is to be made of lysozyme-insulin it will have to be shown that repeated injections do not sensitize to lysozyme. Crystalline lysozyme has been reported to be mildly antigenic.<sup>5</sup> Less pure preparations are reported to be non-antigenic.<sup>6</sup> Upon gently evaporating the heated, alcoholic

\*\* The results for one rabbit injected with protamine-zinc-insulin in the second group of experiments who had convulsions and died are not included in the curve.

<sup>5</sup> Smollens, J., and Charney, J., *J. Bact.*, 1947, 54, 101.

<sup>6</sup> Kigasawa, T., *Ztschr. f. Immunitätsforsch.*, 1928, 57, 146; Wolf, L. K., *ibid.*, 1927, 50, 88.

TABLE I.  
Precipitation of Insulin by Untreated and Treated Lysozyme. (1.0 ml of 0.10% solution of insulin was used in all the tests).

ML of 0.10%-lysozyme (uncorrected for 9.54% moisture)	0.30	0.40	0.50	0.60	0.70	0.80	0.90	Treatment of lysozyme prior to its addition to the insulin
Insulin in supernate	+	+	+	+	+	+	+	
Lysozyme in supernate	—	—	—	—	—	—	—	
mg N in ppt.	0.138	0.185	0.216	0.236	0.237	0.236	0.241	Untreated (N = 17.0% on wet basis). The
% of insulin precipitated	60	80	90	?	100	100	100	insulin contained 14.5% N.
Insulin in supernate	+	+	+	+	+	+	+	
Lysozyme in supernate	—	—	—	—	—	—	—	
mg N in ppt.	0.138	0.180	0.215	0.235	0.238	0.244	0.242	Methylene blue added, used immediately after
% of insulin precipitated	60	77	90	?	100	100	100	mixing.
Insulin in supernate	+	+	+	+	+	+	+	
Lysozyme in supernate	—	—	—	—	—	—	—	
mg N in ppt.	0.112	0.145	0.174	0.206	0.220	0.242	0.261	Methylene blue addition + exposure to day-
% of insulin precipitated	42	53	61	72	70	73	75?	light for 6 days.
Insulin in supernate	+	+	+	+	+	+	+	
Lysozyme in supernate	—	—	—	—	—	—	—	
mg N in ppt.	0.019†	0.069†	0.090	0.136	0.191	0.219	0.250	Dissolved in 75% ethanol, heated for 2 hr in
% of insulin precipitated	?	?	?	?	50	57	64	boiling water bath.
Insulin in supernate	+	+	+	+	+	+	+	
Lysozyme in supernate	—	—	—	—	—	—	—	
mg N in ppt.	0.021†	0.023†	0.063†	0.167	0.206	0.256	0.269	Dissolved in water and autoclaved for ½ hr at
% of insulin precipitated	?	?	?	?	60	83	80	123°C.

\* Supernate, after centrifuging, is opalescent.

† Nitrogen in precipitate is less than in added lysozyme alone, although test for latter in supernatant fluid was negative. This is possibly owing to incomplete sedimentation of the lysozyme-insulin by centrifuging; the opalescence of the supernate suggested that the complex was present in the form of particles too small to be thrown down by the centrifugal force employed.

5 ml. Enough diatomaceous earth (5-7 g) was added to this concentrated urine sample to form a friable powder which was dried at 75-80°C *in vacuo* (7-10 in.) for 16 hours.

The dry powder was transferred to a Waring Blender, 35 ml of  $\text{CHCl}_3$  (U.S.P. grade) added, and the mixture stirred for one minute. The mixture was transferred to an Erlenmeyer flask; then the blender was rinsed with 15 ml of  $\text{CHCl}_3$ , which also was poured into the Erlenmeyer flask. The flask was stoppered with a cork covered with aluminum foil, and the mixture shaken for an hour, then filtered. The residue was washed 3 times with 15 ml portions of  $\text{CHCl}_3$ , pressing out excess solvent each time, and the combined filtrate and washings evaporated on the hot plate to a volume of 1 ml. This last quantity was quantitatively transferred to a 25 ml Erlenmeyer flask and evaporated to dryness by heating in the vacuum oven for one half hour. The dry residue was taken up in one ml of 95% ethanol by shaking for one hour, the alcoholic solution decanted to a clean Erlenmeyer flask, the original flask washed with one ml of alcohol, and the solution together with the ethanol wash dried in a vacuum oven for 30 minutes. Five ml of  $\text{CHCl}_3$  were added to the dry residue, boiled down to one ml, allowed to stand for 30 minutes, decanted into a fresh Erlenmeyer flask, the original flask washed with  $\text{CHCl}_3$ , and the combined solvent evaporated to complete dryness in vacuum oven. Fifty ml of Tyrode's solution were added to the final residue and the flask shaken for one hour. The isolated embryonic duck heart was found to beat in this final solution as regularly and as normally as it did in pure Tyrode's solution.

Known amounts (1-12  $\mu\text{g}$ ) of pure digitoxin (Sandoz) then were added to 200 ml quantities of human urine and the urines were extracted as above. It was found that the final extract produced a "digitalis effect"<sup>2</sup> if two or more micrograms of digitoxin were added to the original 200 ml of urine. Moreover, the

TABLE II.  
Urinary Excretion of Digitoxin in Normal Human Subjects.

Age	First 24 hr			Second 24 hr			Third 24 hr		
	U.V. (ml)	Time of "dig. effect" (min.)†	Tot. amt. dig. excr. ( $\mu\text{g}$ )	U.V. (ml)	Time of "dig. effect" (min.)†	Tot. amt. dig. excr. ( $\mu\text{g}$ )	U.V. (ml)	Time of "dig. effect" (min.)†	Tot. amt. dig. excr. ( $\mu\text{g}$ )
39	1450	21(6)	44	1500	19(8)	60	1700	13(10)	85
31	1720	14(10)	86	2250	24(6)	68	940	19(8)	38
35	2000	20(6)	60	1210	14(10)	60	970	26(4)	19
30	2470	10(12)	144	1050	23(6)	30	520	46(2)	5
24*	1700	12(12)	102	1930	20(6)	58	760	23(6)	23
Avg	1868	15	87	1588	20	55	978	26	34

\* Female.

† Figures in parentheses represent calculated amount of digitoxin ( $\mu\text{g}$ ) in original 200 ml quantity of urine.

<sup>1</sup> Bine, R., Jr., and Friedman, M., *PROC. SOC. EXP. BIOL. AND MED.*, 1948, **69**, 487.

<sup>2</sup> Friedman, M., and Bine, R., Jr., *PROC. SOC. EXP. BIOL. AND MED.*, 1948, **67**, 533.

solution described above to dryness the lysozyme was found to be insoluble in water. This may be taken as an indication of denaturation. While a marked serological change follows denaturation it does not necessarily destroy antigenicity. More study of the antigenic properties of lysozyme seems indicated.

The presence of lysozyme in secretions and tissues<sup>7</sup> suggests a possible precipitating effect on insulin *in vivo*. The solubility of the complex in serum is sufficiently great, however, to make this unlikely.

**Summary.** Crystalline egg lysozyme precipitates insulin. The complex produces a prompt

lowering of the blood-sugar level in rabbits. Precipitates formed by mixing insulin with lysozyme which has been heated, or treated photodynamically so that 90 to 100% of its lytic activity on *M. lysodeikticus* is destroyed, but about 60% of its precipitating power for insulin is left, produce a hypoglycemic response which, up to 7 hours after injection, is intermediate between that resulting from the injection of insulin in solution and that following administration of protamine-zinc-insulin.

I wish to record my appreciation to Mr. E. K. Wolfe of the Biochemical Control Department of Sharp and Dohme, Inc., Glenolden, Pa., for performing the animal experiments.

<sup>7</sup> Fleming, A., and Allison, V. D., *Brit. J. Exp. Path.*, 1922, **3**, 252.

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## Renal Excretion of Digitoxin in Man Following Oral Administration.\* (17470)

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The amount of digitoxin excreted in the urine of human subjects receiving the drug has not been determined, primarily because of previous inability to measure minute amounts of any type of digitalis glycoside in biological media. However, the discovered sensitivity of the embryonic duck heart preparation<sup>1</sup> to minute amounts of digitoxin when present in Tyrode's solution and human serum offered

the possibility that this method also could be used for the quantitative detection of digitoxin excreted in the urine of patients receiving the drug. The preliminary results of this study are reported herein.

**Methods.** A human urine sample of 200 ml was boiled down to approximately 50 ml on a hot plate and thereafter more slowly evaporated until the volume was reduced to

TABLE I.  
Relationship Between Initial Concentration of Digitoxin in Human Urine (200 ml) and Time of Occurrence of "Digitalis Effect" in Final Extract.

Amt. dig. added ( $\mu$ g)	No. of extracts assayed	Avg time, "dig. effect" (min.)	S.E. of mean (min.)
1	5	—	—
2	12	47	2.28
3	10	35	1.82
4	11	27	1.45
6	9	22	1.14
8	10	17	0.73
10	5	14	0.35
12	6	11	0.20

\* Aided by grants from The Life Insurance Medical Research Fund, The United States Public Health Service, and the Sandoz Chemical Works.

TABLE I.  
 Tests for Adrenotrophic Activity in Human Urine.

Reference	Source	Preparation	Total vol. per animal, ml	Assay method	Adreno- trophic activity
DeBoissezon <sup>1</sup> (1936)	Pregnancy	Untreated	16	Incr. in adrenal wt and cholesterol in intact guinea pig	Yes
Paschkis, Rakoff, and Cantarow <sup>2</sup> (1942)	Normal	70-86% ethyl alcohol precipitate	Not stated	Incr. in adrenal wt in intact 2-day-old chicks and 4-day-old rats	Yes
Jones and Bucher <sup>3</sup> (1943)	Normal	Fractional ammonium sulfate precipitates	200-440	Increase in adrenal wt of hypophysectomized rat	No
Blumenthal <sup>4</sup> (1945)	Pregnancy, normal female	Untreated, fresh and preserved	4-16	Incr. mitosis in adrenals of intact guinea pig	Yes
Reiss, Peglar, and Gollas <sup>5</sup> (1946)	Pregnancy	1. Untreated 2. Benzoic acid adsorbed 3. Pierie acid ppt.	Not stated Not stated 25	Disappearance of sudan- ophobe zone in adrenal of hypophysectomized rat	1. No 2. No 3. Yes
Williamson <sup>6</sup> (1946)	Normal female	1. Untreated 2. Dialyzed 3. Dialyzed, acetone pre- cipitated 4. Dialyzed pervaporated and acetone precipitated	4 4 120-460 30	Incr. in adrenal wt in intact rats Ascorbic acid depletion of adrenals of hypo- physectomized rat	1. Yes 2. Yes 3. Yes 4. Yes
Cooke, Graetzer, and Reiss <sup>7</sup> (1948)	Normal	Permutit adsorbed	250	Ascorbic acid depletion of adrenals of hypo- physectomized rat	Yes

presence of adrenotrophic activity in about 25 ml of urine of normal and pregnant humans. Since our work was completed, Cooke and associates<sup>7</sup> have reported positive reactions for adrenotrophin in concentrates representing 250 ml of normal urine, prepared by an adsorption technic.

<sup>2</sup> Paschkis, K. E., Rakoff, A. E., and Cantarow, A., *Endocrinology*, 1942, **30**, 523.

<sup>3</sup> Jones, G. E. S., and Bucher, N. L. R., *Endocrinology*, 1943, **32**, 46.

<sup>4</sup> Blumenthal, H. T., *J. Lab. and Clin. Med.*, 1945, **30**, 428.

<sup>5</sup> Reiss, Max, Peglar, Harold, and Golla, Y. M. L., *Nature*, 1946, **157**, 413.

<sup>6</sup> Williamson, M. B., *Proc. Soc. Exp. Biol. and Med.*, 1946, **63**, 191.

<sup>7</sup> Cooke, D. S., Graetzer, E., and Reiss, M., *J. Endocrinol.*, 1948, **3**, lxxxix.

Because of these conflicting results and because of some claims that the presence of adrenotrophin in body fluids in Cushing's syndrome is of etiologic significance, we have reinvestigated this problem using several different methods of concentrating the urine, and have placed particular emphasis on the study of urine from such pathologic states as may conceivably be associated with extremes of adrenotrophic activity. Although in general, our results have been negative, we are reporting them briefly, since they may be of some interest and of some use to others interested in the clinical-laboratory aspects of pituitary-adrenal disorders.

*Material and methods.* Adrenotrophic activity was determined by the ascorbic acid depletion method described by Sayers, Sayers



time of occurrence of the "digitalis effect" in the duck hearts was dependent upon the amount of digitoxin added. Thus, standards (Table I) were obtained. Such standards were found to be necessary because of the significant but relatively fixed loss of digitoxin in the extraction of such large quantities of urine.

For the assay of urinary excretion of digitoxin, control urine samples (200 ml) were obtained from 4 young males and one female subject (average age 32 years). These were extracted and tested on the duck hearts as described above and found to produce no effect. Each subject then was given 1.2 mg of digitoxin by mouth within a period of 6 hours. Urine collections subsequently were obtained. The daily volumes were measured, and then 2 separate 200 ml samples of each were extracted and assayed on 6-8 duck hearts. The average time of occurrence of the "digitalis effect" of the 2 samples was compared to the standard values. In this manner the amount of digitoxin originally present in a 200 ml quantity of urine could be approximated. The total daily excretion then was calculated by multiplying the amount of digitoxin in the 200 ml quantity of urine, by the total daily urine volume/200.

**Results.** Each of the 5 subjects (Table II) excreted greatly varying amounts of digitoxin in his urine after the oral ingestion of 1.2 mg of the drug. During the first 24 hours after ingestion an average of approximately 87  $\mu$ g (Range: 44-144) or 7% of the administered dose could be detected in the urine. The second day's urine of each subject contained somewhat less digitoxin (average: 55  $\mu$ g). The third day's urine of 4 subjects tested contained appreciably less digitoxin (average: 21  $\mu$ g). These results indicate that approximately 14% of the amount of digitoxin administered to these 5 subjects could be recovered in the urine collected the first 3 days after its administration.

**Summary.** The quantitative detection of orally administered digitoxin in the urine of subjects receiving the drug has been accomplished. Approximately 14% of the given dose is excreted in the urine of normal subjects during the first 72 hours after its oral ingestion.

The authors wish to express their indebtedness to Maude Gardner and Nancy Bryant for their invaluable assistance.

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## Adrenotrophic Activity of Human Urine.\* (17471)

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The ability to determine adrenocorticotrophic hormone in body fluids of man would be a valuable aid in extending our knowledge of pituitary-adrenal physiology. Although there have been previous reports dealing with the presence of adrenotrophin in urine, the results (summarized in Table I) are conflict-

ing. Part of this confusion arises from the failure of some of the investigators to use hypophysectomized test animals, an omission which unfortunately invalidates their conclusions. The remaining confusion apparently cannot be so explained. For example, Jones and Bucher,<sup>3</sup> using a specific assay method, found no adrenotrophic activity in 200 to 440 ml of urine of normal persons, while Reiss and associates<sup>2</sup> and Williamson<sup>6</sup> have reported the

\* Abridgment of thesis submitted by Dr. Locke to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Medicine.

† Deceased.

<sup>1</sup> deBoissezon, P., *Bull. d'histol. appliq. a la physiol.*, 1936, 13, 129.

TABLE II.  
Assays for Adrenotrophic Activity in the Urine of Various Endocrinopathies.

No. of patients	Diagnosis	No. of assays	Adrenotrophic activity		
			Positive (L—R >25 mg)	Doubtful (L—R = 20-25 mg)	Negative (L—R <20 mg)
4	Cushing's syndrome, Adrenal cortical hyperplasia	9	1	3	5
2	Hypopituitarism	2	0	1	1
1	Addison's disease	1	0	0	1
2	Sexual and somatic precocity	2	1	0	1
1	Natural menopause	1	1	0	0
3	Surgical menopause	3	1	0	2
1	Ovarian dwarfism	1	0	0	1
1	Pregnancy 5 mo.	1	0	1	0
1	Pituitary tumor	1	1	0	0

be recovered. Concentrates equivalent to 15% of the total 24-hour specimen, obtained from menopausal and pregnant women, states in which urinary adrenotrophin had been previously detected, gave negative results.

*Acetone precipitates of urine.* The precipitation of urine by means of acetone and the extraction of the precipitate yield fairly good recoveries of urinary gonadotrophin (Frank and associates<sup>21</sup>). This method was investigated extensively for adrenotrophic activity. Twenty-four-hour urine specimens were collected from 7 normal subjects (4 females and 3 males) and 10 tests were carried out. Concentrates ranging from 2.5 to 23% of the total 24-hour specimen were injected per rat. Concentrates equivalent to 2.5 to 10% of the total specimen gave negative results. However, the equivalent of from 10 to 23% of the specimen per rat yielded positive L—R values (more than 25 mg) in 2 instances, borderline responses (L—R of 20 to 25 mg) in 2 instances, and negative responses in 2 instances. Thus, while far from consistent, these results indicate at least that an amount equivalent to 10% or more of the 24-hour specimen was necessary to induce positive responses.

Since adrenotrophic activity could not be

demonstrated consistently in normal urine, tests were performed on urines which might conceivably be richer in adrenotrophin, that is, in certain endocrine disorders. Among the endocrinopathies which might be associated with extremes of adrenotrophic activity, we tested the urines of patients with Cushing's syndrome, hypopituitarism, Addison's disease, in the menopause, and with a few miscellaneous disorders (pituitary tumor, sexual and somatic precocity and ovarian dwarfism). All tests were performed with amounts of urine representing 10% or more of the entire 24-hour specimen. The results of these assays are summarized in Table II.

*Comment.* The attempts to demonstrate adrenotrophic activity in human urine of both normal and pathologic states have been generally unsuccessful. We have been unable to confirm the presence of adrenotrophin with consistency, although occasional specimens have indeed given positive results. While it may be possible that adrenotrophin is either not excreted in the urine or excreted in biologically inactive form, the few occasional positive results encountered by us as well as by other investigators would seem to overrule this possibility. Another possibility is that adrenotrophin is excreted in biologically

and Woodbury<sup>8</sup> modified by: (1) the use of hypophysectomized male rats of the Wistar strain rather than the Sprague-Dawley, and (2) the injection of test material into the jugular vein or peritoneum, rather than the tail vein. In most instances 5 rats were used for each sample of urine. Ascorbic acid concentration of the rat adrenal glands was determined according to the procedure described by Roe and Kuether,<sup>9</sup> adapted for use with tissue. The solutions were read in a Coleman, Jr. spectrophotometer at 510  $\mu$  and the adrenotrophic activity was expressed in terms of the reduction of ascorbic acid concentration (milligrams per 100 g fresh tissue) in the right gland, removed one hour after injection, as compared to the left gland, removed prior to the administration of the urine preparation. This calculation is subsequently designated as Left—Right, or L—R.

Urine was obtained from a variety of normal and abnormal subjects and prepared by one of the following methods: (1) fresh unconcentrated urine, or fresh dialyzed urine; (2) urine dialyzed and pervaporated to one-fifth volume, after which it was precipitated by means of acetone (Williamson,<sup>6</sup>); (3) ultrafiltration of urine through a collodion membrane at 60 to 80 lb pressure, after which the membrane was dissolved and the residue was extracted with 0.1 N sodium hydroxide and neutralized (Gorbman<sup>10</sup>); and (4) urine precipitated by 4 volumes of acetone at pH 3.5 and the precipitate extracted with 0.1 N sodium hydroxide, neutralized and dialyzed (Frank and associates<sup>11</sup>).

In order to obtain control values for comparison with experimental values, solutions containing no adrenotrophic activity (0.9% saline solution; distilled water) were administered to 25 hypophysectomized rats in volumes of 1 to 10 ml according to the pro-

cedure described previously. The mean concentration of ascorbic acid in the right gland was found to be higher than the left by  $4 \pm 3.2$  mg, or a mean L—R of  $-4 \pm 3.2$ . These results are much more variable than those reported by Sayers and associates<sup>8</sup> which indicate no difference in ascorbic acid concentration between left and right glands.

Ordinarily the urine or urinary concentrate was administered to 5 rats, but owing to toxicity not all the animals survived each experiment. Only those results in which 2 or more animals survived so that a mean value could be determined are included. Because of the variability of the animals, already mentioned, no mean L—R value less than 25 mg was considered significantly different from the controls, this value representing the minimal level for a test of significance between means. That toxicity of the concentrate did not impair the action of adrenotrophin was assured by dissolving pure adrenotrophin in the concentrate and observing the response as compared with the same amount of hormone in saline. Under these conditions, no difference in the L—R values was observed.

*Results. Fresh, unconcentrated or dialyzed urine.* Williamson<sup>6</sup> reported that 4 ml of fresh urine possessed adrenotrophic activity as measured by the increase in adrenal weight in the intact rat. Urines obtained from 3 normal persons, one patient with Cushing's syndrome and one patient with somatic and sexual precocity were inactive when tested in hypophysectomized rats in amounts of from 2 to 10 ml of urine per rat. Thus, the results obtained with intact rats would appear to be nonspecific responses.

*Dialysis and pervaporation method of Williamson.* Fresh mixed female urine prepared by dialysis, pervaporation and acetone precipitation was reported by Williamson to induce an L—R value of 145 mg when administered in an amount of 30 ml equivalent of urine. This method was repeated using from 14 to 88 ml equivalent of urine with entirely negative results.

*Ultrafiltration method.* The method described by Gorbman<sup>10</sup> for the recovery of urinary gonadotrophin was tested to determine whether adrenotrophin, if present, could also

<sup>8</sup> Sayers, M. A., Sayers, George, and Woodbury, L. A., *Endocrinology*, 1948, **42**, 379.

<sup>9</sup> Roe, J. H., and Kuether, C. A., *J. Biol. Chem.*, 1943, **147**, 399.

<sup>10</sup> Gorbman, Aubrey, *Endocrinology*, 1945, **37**, 177.

<sup>11</sup> Frank, R. T., Salmon, U. J., and Friedman, Reuben, *Proc. Soc. Exp. Biol. and Med.*, 1935, **32**, 1666.

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active form but as a degraded part of the protein molecule. Such a possibility might explain why most of the methods used, designed to concentrate the urinary protein, do not yield adrenotrophic activity with consistency.

Assuming that adrenotrophin is excreted in human urine, the problem of its detection might be resolved in two ways. The first approach would be to increase greatly the sensitivity of the bioassay procedure so as to permit the use of fresh urine. Were it possible to obtain positive results with a few milliliters of fresh urine, it would then be relatively simple to work out systematically a technic for the concentration and purification of the hormone from urine. However, since the present Sayers' assay procedure is already extremely sensitive (in our hands a minimum of 0.8  $\mu$ g of pure adrenotrophin was capable of inducing significant L—R values) it seems quite unlikely that any new assay

method sufficiently sensitive to permit the use of fresh urine will be soon available.

The alternative approach would be to continue the empirical attempts to purify and concentrate the urine in the hope that a method will be found to permit the administration of large volumes of urine without toxicity to the test animals or appreciable loss of adrenotrophic activity. As such, the method described by Cooke and associates may represent a solution.

*Summary.* Using a specific and sensitive assay procedure, we have tested the urine of normal persons and of patients having a variety of disease states for its adrenotrophic activity. Tests using both fresh urine and urine concentrates prepared in three different ways were carried out. Contrary to previous reports, no adrenotrophic activity could be consistently demonstrated.

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## Plasma Levels of Radioactive Iodine ( $I^{131}$ ) in Human Tracer Studies. (17472)

HILDA S. ROLLMAN, AND DONALD W. PETIT (Introduced by Paul Starr)

*From the School of Medicine, University of Southern California, and Endocrine Clinic, Los Angeles County Hospital.*

The purpose of the studies reported here was to examine the post ingestion plasma radioiodine curves in euthyroid, hypothyroid, and hyperthyroid subjects as a first step toward analysis of the various factors regulating the levels of such plasma iodine curves.

*Methods.* Patients were selected after previous clinical and laboratory study had established the presence of a euthyroid, hypothyroid or hypothyroid state. All were in an untreated state. They were given 100 microcuries of radioiodine by mouth before breakfast. The dosage was calculated on the basis of the shipping tag from Oakridge Laboratories. No carrier iodine was added. Each dose was given with 50 cc of distilled water and followed by two 50 cc portions of water to wash the container and mouths of the subjects. Blood specimens were drawn

at 30 minutes, 1, 3, 6, 12, 24, and 48 hours; the blood was oxalated and centrifuged at 2500 rpm for 10 minutes. Then 0.2 cc of plasma was pipetted into shallow cups containing one drop of phenolphthalein, 0.2 cc of 0.75 N NaOH and 0.2 cc of detergent (Orvus). Duplicate samples were dried under an infra-red lamp and counted with a bell type Geiger Müller tube (Cyclotron Specialties Co., Model 410-A) and counter (Technical Associates Model GS4). Duplicate specimens of reagents alone, exclusive of plasma, were checked for radio-contamination. The counts were corrected for decay with reference to the day of ingestion. Eleven cases of untreated myxedema and 14 non-myxedematous cases (5 hyperthyroid and 9 euthyroid) were so studied.

*Results.* When the data for the entire 25

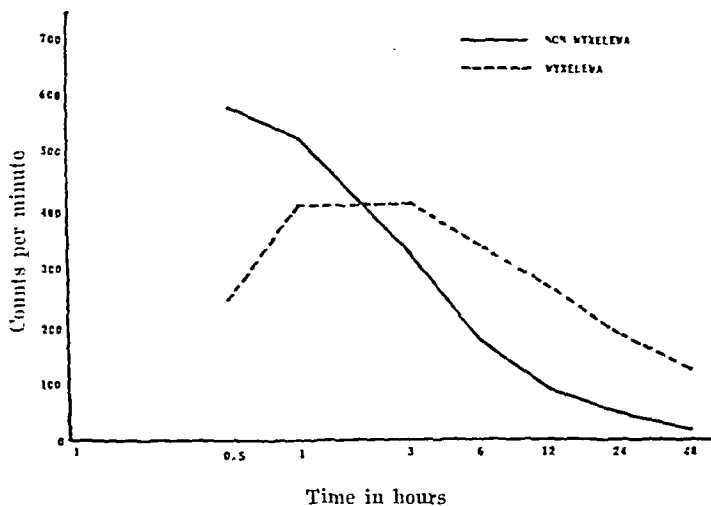
Plasma Levels of  $I^{131}$ 

FIG. 1.

Combined plasma curves of the geometric means of the entire myxedematous and non-myxedematous groups.

patients were analyzed, two curves could be obtained, one for the myxedematous and one for the non-myxedematous patients.\* Fig. 1 shows these curves; here the geometrical mean (calculated by probit analysis) of counts per minute is plotted on an arithmetical scale against time on a logarithmic scale.

The data from which these curves were obtained are shown in Table I.

**Discussion.** The major differences noted between these 2 groups of patients (myxedematous and non-myxedematous) may be listed as follows: 1. An early, high peak in the plasma radioactivity of the non-myxedematous group. 2. A later, lower peak in the plasma radioactivity of the myxedematous group. 3. A slower decrease in radioactivity during the first 48 hours in the myxedematous group. These differences appear valid; it seems unlikely that the effect of plasma volume or pre-existing iodine content of plasma would be the cause of these changes. It is more reasonable to suppose that the variations in these two curves are caused by: (a) different rates of absorption of radioiodine from the gastro-intestinal tract, (b) varying rates of

excretion of radioiodine by the kidney, (c) speed of formation and storage of thyroid

TABLE I.

Plasma Radioactivity, as Counts Per Minute of the Three Groups of Patients Studied. The individual patient's curve is read horizontally under the time intervals shown.

30"	1'	3'	6'	12'	24'	48'
Euthyroid						
398	350	218	120	62	10	0
460	372	231	129	62	19	0
518	398	291	170	71	25	0
600	446	313	172	90	18	4
635	480	363	194	91	31	7
671	530	363	231	118	45	20
704	638	443	247	128	70	24
731	676	499	248	128	121	63
860	822	636	272			
Hyperthyroid						
280	400	148	63	18	16	42
551	417	258	125	57	57	48
555	496	321	190	136	137	195
813	911	385	225	148	183	218
		580	289	198	184	
Myxedema						
75	212	296	252	184	91	19
88	223	319	253	197	116	31
158	272	349	291	199	117	50
275	345	373	304	202	139	121
312	384	381	393	213	163	125
315	459	396	336	220	216	167
336	465	416	346	274	247	190
347	522	432	358	307	278	199
397	541	493	368	320	325	205
400	568	541	400	385	364	278
542	592	543	565	475		

\* The authors are indebted to Frederick J. Moore, M.D., for the statistical analysis of the data.

active form but as a degraded part of the protein molecule. Such a possibility might explain why most of the methods used, designed to concentrate the urinary protein, do not yield adrenotrophic activity with consistency.

Assuming that adrenotrophin is excreted in human urine, the problem of its detection might be resolved in two ways. The first approach would be to increase greatly the sensitivity of the bioassay procedure so as to permit the use of fresh urine. Were it possible to obtain positive results with a few milliliters of fresh urine, it would then be relatively simple to work out systematically a technic for the concentration and purification of the hormone from urine. However, since the present Sayers' assay procedure is already extremely sensitive (in our hands a minimum of 0.8  $\mu$ g of pure adrenotrophin was capable of inducing significant L—R values) it seems quite unlikely that any new assay

method sufficiently sensitive to permit the use of fresh urine will be soon available.

The alternative approach would be to continue the empirical attempts to purify and concentrate the urine in the hope that a method will be found to permit the administration of large volumes of urine without toxicity to the test animals or appreciable loss of adrenotrophic activity. As such, the method described by Cooke and associates may represent a solution.

*Summary.* Using a specific and sensitive assay procedure, we have tested the urine of normal persons and of patients having a variety of disease states for its adrenotrophic activity. Tests using both fresh urine and urine concentrates prepared in three different ways were carried out. Contrary to previous reports, no adrenotrophic activity could be consistently demonstrated.

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*Results.* When the data for the entire 25

TABLE I.  
Adaptation and Its Effect on Hours Survival at Low Air Pressure.

No. rats	Time at 268 mm Hg, 14°C,* hr	Time at 162 mm Hg, 14°C,* hr	Time at 162 mm Hg, 29-31°C, hr	Remarks
6 ♂	4	5		All lived
2 ♀	2	8		One died at end
3 ♂	4	2	3	All lived
7 ♀	2	1.5	4.5	Two died
Controls				
5 ♂ and ♀		.12 ± .06		All died
1 ♀		1.0		Removed alive
17 ♂			.20 ± .18	All died
24 ♀			.25 ± .20	" "

\* Temperature range 11° to 15°C.

They were continuously ventilated at the rate of 2 liters of air per rat per minute. The flow rate was measured at 760 mm Hg. The chambers could be operated either within a refrigerator or at room temperature. The experimental rats were Long-Evans strain, unfasted, male and female, old and young adults.

**Results.** Control rats were placed within the chambers at various temperatures (14-31°C) and the air pressure reduced within 3 minutes to 162 mm Hg. Within 6 to 15 minutes these rats developed convulsions and died. The "adaptation" experiments were conducted with the chambers placed within a refrigerator at 14°C (range 11° to 15°C) and the pressure reduced within 90 seconds to 268 mm Hg and maintained at that pressure for 2-4 hours. Then the pressure was further reduced to 162 mm Hg (14°C) and these conditions were maintained for 1.5-2 hours. At this point some of the chambers were removed to room temperature (29-31°C) and others were left within the refrigerator, the air pressure in both sets of chambers was still maintained at 162 mm Hg (oxygen pressure 32 mm Hg). After 4-6 additional hours under these conditions, 17 out of 20 rats remained alive and they were returned to sea level for further observation. These rats therefore endured for 5-8 hours anoxic conditions which killed 98% of controls in 10-15 minutes and this high degree of tolerance was developed in a 3-4 hour adaptation period.

The permanency of this adaptation was tested by the following experiment. Two rats were exposed to cold and anoxia as outlined in the above paragraph. The air atmosphere

at 30°C was then replaced by 150 mm of pure oxygen, which is approximately equivalent to the sea level pressure of oxygen. After 4 hours the oxygen atmosphere was replaced by air at 162 mm and the animals succumbed in 12 and 15 minutes, essentially the same as unadjusted controls. Therefore, only 4 hours of oxygenation was adequate to destroy the tolerance which these rats had developed.

Of 20 rats adapted to anoxia as outlined above, only 3 died when exposed continuously to air at 162 mm for 5-8 hours (Table I). Of 46 control rats at the same pressure, 45 died in 4-27 minutes (0.06-0.45 hour) and one lived for one hour (Table I). Low environmental temperature was important in developing the tolerance of the adapted group, but the control rats died at a variety of temperatures.

The adapted animals were allowed to recover at sea level and room temperature. Their rectal temperature was 19-20°C if the entire exposure to cold and anoxia had been at 14°C. If the last 4-6 hours of anoxia was at room temperature, the rectal temperature was 1-2°C above room temperature (29-31°C). These animals were quite refractory to mechanical stimuli for the first 5-15 minutes following the anoxia period. However, within 2-3 hours, they would often eat and drink and within 24 hours they appeared normal. All of these animals were still alive one month after the experiment.

The eyes of the adapted animals showed striking changes. After 3 or more hours at an air pressure of 168 mm Hg the eyes were clouded by severe cataracts. Examinations of the lens directly by excision and indirectly by



hormone, (d) re-entry of radioiodine into the blood stream in a protein bound form. How much of a role each one of the above factors might play in the ultimate derivation of the plasma curve requires further study.

While this work was in progress McConahey *et al.*<sup>1</sup> published the results of a similar study of serum radioactivity in hyper-, hypo-, and euthyroid individuals, after ingestion of  $I^{131}$ . They reported experiments on 31 patients divided as follows: 17 with hyperthyroidism, 9 euthyroid, and 6 myxedematous (one patient was studied twice, once while euthyroid and then after a "thyroidectomizing" dose of  $I^{131}$ ). Their patients did not receive similar doses of radioiodine but rather there was a range from 0.1 to 100 millicuries of the drug. McConahey *et al.*<sup>1</sup> felt that there was little if any disturbing radiation effect on the plasma curves from the large doses given to some patients.

The principal finding of difference in McConahey's work from the work presented here, is the failure of our figures to differentiate hyperthyroid and euthyroid individuals. It seems reasonable to suppose that hyperthyroid indi-

viduals would show a different rate of increment and decrement of radioiodine in blood after an oral dose than would euthyroid individuals. The differences obtained here, however, were not sufficient to be used for diagnostic purposes and probit analysis of the data would not significantly separate the two groups in the time periods studied.

**Summary.** Curves of plasma radioactivity were determined for 25 patients after the ingestion of 100 microcuries of radioiodine. The patients were divided into 11 cases of myxedema, 5 cases of hyperthyroidism and 9 euthyroid individuals. The curves were constructed from plasma samples drawn at 30 minutes, 1, 3, 6, 12, 24, and 48 hours. It was not possible to statistically separate, at these time intervals, the curves of the hyperthyroid and euthyroid individuals. The curves of the myxedematous patients, however, were significantly different from those of the non-myxedematous patients; thus the myxedematous curves show a later peak level, a lower peak level and a fall more delayed than do the curves of the non-myxedematous patients.

<sup>1</sup> McConahey, W. M., Keating, F. R., Jr., and Power, M. H., *J. Clin. Invest.*, 1949, **28**, 191.

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## Acute Adaptation of Rats to Very Low Oxygen Pressure.\* (17473)

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In a recent publication<sup>1</sup> the author reported evidence of depressed activity of the thyroid gland in anoxic rats. This thyroid depression may act as an adaptive change which would aid the accommodation to anoxia. Himwich<sup>2</sup> called attention to the evidence of Barach and others<sup>3,4</sup> which indicated that thyroidec-

tomy increases the tolerance of rats to anoxia. Therefore, if anoxia were to result in a degree of functional thyroidectomy, this latter condition should increase the tolerance to more severe anoxia.

The experiments described here indicate that if normal rats are first exposed to anoxia for 2 or more hours at a low temperature, they can then withstand severe anoxia as readily as has been reported for surgically thyroidectomized rats.<sup>5,4</sup>

**Method.** The low pressure chambers were constructed from 9 liter vacuum desiccators.

\* This investigation was aided by grants-in-aid from the National Research Council Committee on Endocrinology and the Atomic Energy Commission.

<sup>1</sup> Van Middlesworth, L., *Science*, 1949, **110**, 120.

<sup>2</sup> Himwich, H. E., personal communication, 1949.

<sup>3</sup> Barach, A. L., Eckman, M., Molomut, N., *Am. J. Med. Sci.*, 1941, **202**, 336.

<sup>4</sup> Streuli, H., *Biochem. Z.*, 1918, **87**, 359.

## Function of Egg White in Embryonic Development. (17474)

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In an attempt to decide whether or not the yolk or the egg white is the carrier of a growth factor transmitted through the egg from the hen's feed to the chick, the experimental technic described here was devised. In the past it has been assumed that egg white has a purely nutritional function in the growth and development of the chick embryo, but no experimental evidence exists to support this supposition.

The yolk also furnishes food, but in addition the yolk-sac serves as an organ for the digestion and assimilation of food by the chick embryo. This function of the yolk-sac indicates a structural relationship between the embryo and the yolk.

In this experiment fertile hen's eggs from crosses of Rhode Island Reds and White Leghorns were used. A circular window approximately  $\frac{1}{4}$  inch in diameter was bored in the small end of each egg and the egg-white was removed into a glass ground-joint wash bottle by suction. To combat bacterial infection 0.1 cc of an aqueous solution containing 3000 I.U. of penicillin and 3.3 mg of streptomycin was injected into each of the eggs so treated. The egg white from another fertile egg was then transferred by air pressure from the wash bottle into the egg from which the egg white had previously been removed. When one egg white was not sufficient to completely refill

the shell, that from another fertile egg was added. Thus, at times egg white from more than 1 egg was used for replacement in a single egg.

After the egg was completely filled with egg white, the window in the shell was closed with a strip of scotch tape and the egg placed into an incubator for 3 weeks. At intervals of 7 days, the condition and development of the chick embryo was observed by candling the eggs.

In the first successful series, 12 such eggs were prepared; of these 3 chicks hatched which were normal in every respect. Of the rest of the embryos, two lived 2 days, one 4, two 6, one 7, one 13, one 15 and one 21 days.

These are probably the first live chicks produced after such drastic treatment. This experiment demonstrates that the chief function of the white of the hen egg is mainly to furnish food for the developing embryo.

It has been previously held that the two chalazae serve to orient the embryo in a proper position for hatching by keeping the yolk more or less fixed. In these experiments, however, the two chalazae were removed with the egg white and thus the position of the developing embryo depended upon gravity. Further work is in progress on the nutrition of the chick embryo employing this technic.

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## Distribution of Vitamin B<sub>12</sub> in Natural Materials.\* (17475)

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The increasing use of vitamin B<sub>12</sub> in the treatment of human macrocytic anemias and in certain natural rations for farm animals makes the knowledge of the distribution of this vitamin in biological materials of real im-

portance. A rat assay for vitamin B<sub>12</sub>, based on the fact that thyroid active materials increase the requirements for this factor, has been used with considerable success in our laboratory.<sup>1,2</sup> With the availability of pure vita-

the slit lamp<sup>†</sup> showed a well developed sub-capsular cataract. Seventy percent (14 cases) of the severely anoxic rats developed cataracts during the period of anoxia but all of the cataractus lenses became clear within 60-120 minutes when the animals were allowed to recover at sea level.

*Discussion.* The acute adaptation to anoxia described here differs from the usual chronic acclimatization to high altitude. In the latter the subjects are said to live fairly normal lives (reviewed by Van Liere<sup>5</sup>) and the acclimatizing process requires weeks to develop<sup>6</sup> although it probably requires generations to become complete.<sup>7</sup> Furthermore, on return to sea level condition, the loss of true acclimatization is slow.<sup>8</sup> In our experiments the animals exhibited a very low level of existence but their tolerance to extreme anoxia developed within a few hours. On return to sea level oxygen pressure the adaptation was lost within a few hours or about as rapidly as it had developed.

This acute adjustment to severe anoxia may involve some or all of the following factors: (a) reduction of body temperature, (b) reduction of metabolic rate, (c) reduction of thyroid activity, (d) anaerobic metabolism. Evaluation of these possible factors must await additional experimental investigation.

It is possible that the adjustment to anoxia as illustrated in this report could have no significant relation to the thyroid gland, but in view of recent work<sup>1</sup> anoxic suppression of the thyroid appears to offer a plausible explanation for the acute adjustment to severe anoxia.

† These slit lamp observations were made by Dr. J. W. McKinney, Department of Ophthalmology, University of Tennessee College of Medicine.

<sup>5</sup> Van Liere, E. J., *Anoxia, its effect on the body*, Univ. of Chicago Press, 1942, p. 140-156.

<sup>6</sup> Houston, C. S., and Riley, R. L., *Am. J. Physiol.*, 1947, **149**, 565.

<sup>7</sup> Monge, C., *Acclimatization in the Andes*, Johns Hopkins Press, 1948 (translated by D. F. Brown).

<sup>8</sup> Norton, E. F., *The Fight for Everest*, London, 1925.

<sup>9</sup> Britton, S. W., and Kline, R. F., *Am. J. Physiol.*, 1945, **145**, 190.

Britton and Kline<sup>9</sup> investigated the effect of temperature on survival of asphyxiated adult rats. Their results demonstrated that animals being asphyxiated in sealed chambers survived 2-3 times longer at 16-18°C than at a temperature of 31-32°C.

Previous investigators<sup>10-12</sup> have increased the tolerance of rats to anoxia by the administration of antithyroid drugs for 8 or more days preceding the low pressure exposure. In the experiments of Barach *et al.*<sup>9</sup> thyroidectomized rats were found to withstand 160 mm Hg for 2 hours (temperature not reported). However, Leblond<sup>12</sup> showed that such thyroidectomized rats must be well fed in order to withstand severe degrees of anoxia. The adapted animals described in the present work easily tolerated the severe anoxic conditions reported for hypothyroid or thyroidectomized rats.

Previous investigators<sup>13,14</sup> have observed cataracts from anoxia. The animals of Bellows and Nelson<sup>13</sup> were exposed to 225 mm until 50 percent of the rats were dead, after which 10% of those surviving had cataracts. Comparison of previous results with these reported here shows that in our experiments the anoxia was more severe, a greater number of rats survived the experiment, and more animals developed cataracts.

*Conclusion.* Rapid adaptation occurs in adult rats exposed to anoxia at low temperatures. This enables the animals to tolerate extreme anoxia (32 mm Hg pressure of O<sub>2</sub>) at various temperatures (14-31°C) for a period more than 40 times longer than unadapted controls.

<sup>10</sup> Gordon, A. S., Goldsmith, E. D., Charipper, H. A., *Science*, 1944, **99**, 104.

<sup>11</sup> Hughes, A. M., and Astwood, E. B., *J. Aviat. Med.*, 1944, **15**, 152.

<sup>12</sup> Leblond, C. P., *Proc. Soc. Exp. Biol. and Med.*, 1944, **55**, 114.

<sup>13</sup> Bellows, J., and Nelson, D., *Arch. Ophthalm.*, 1944, **31**, 250.

<sup>14</sup> Biozzi, G., *Graef's Arch.*, 1935, **133**, 423. (*Eng. Abst. Amer. J. Ophthalm.*, 1935, **18**, 692.)

period. At the end of this time supplements are administered for another 2-week period during which time the growth response is followed. The vitamin B<sub>12</sub> content of the sample is calculated through the use of a standard growth curve plotted from data obtained with graded levels of crystalline vitamin B<sub>12</sub>.<sup>2</sup>

Five rats were used for each assay and an average of the growth response for these rats was taken. In most cases the material to be tested was added on a percentage basis to the basal ration and the vitamin B<sub>12</sub> content was calculated from the total food consumed. In other cases, due to the nature of the material, the supplement was given in separate food containers and the amount consumed was measured directly. The results are summarized in Table I.

Average growth responses on the basal ration during the 2 week assay period have been found for a large number of animals to be 35 g ( $\pm$  5 g). The daily administration of 0.1  $\mu$ g of vitamin B<sub>12</sub> either orally or by injection, increased the growth response to 65-66 g. Since Hall, *et al.*<sup>3</sup> showed that incubation of vitamin B<sub>12</sub> with gastric juice increased the hematopoietic activity in pernicious anemia when administered orally, a similar treatment was tried for the rat. No effect on the activity of vitamin B<sub>12</sub> was noted after incubation with human gastric juice.

Thymidine has been found to be capable of replacing vitamin B<sub>12</sub> in the nutrition of *Lactobacillus lactis*.<sup>4,5</sup> Results presented in Table I show that this compound has no activity in the rat assay. Fresh tomato juice, which has been found to contain the TJ factor required by *Lactobacillus lactis* Dorner,<sup>6</sup> was likewise found to be inactive. Furthermore, a combination of thymine, uracil, adenine, guanine, rutin and hesperidin produced no growth response. It appears, therefore, that the rat

assay is rather specific for vitamin B<sub>12</sub>.

Two samples of yeast autolysates, U2 and Z7, supplied by Dr. C. N. Frey, Standard Brands, Inc., were found to be completely inactive. A sample of "slops" from streptomycin production was found to be very high in vitamin B<sub>12</sub>. The regular commercial condensed fish solubles showed high activity while a sample of herring stickwater was not as active.

Sheep rumen contents were very active, which suggests a synthesis of this vitamin within the rumen. Milk, cheese, meats and egg yolk were found to be relatively good sources of the vitamin. Dried soybean sprouts, barley, fresh potatoes, beans, cabbage, green peas, alfalfa leaf meal, and fresh grass contained no significant amount.

The vitamin B<sub>12</sub> content of beef kidney was found to be as high as that of beef liver. This is in agreement with previous results obtained on the vitamin B<sub>12</sub> content of liver and kidneys from rats fed different rations.<sup>7</sup> The higher values obtained with the two liver powder preparations of the VioBin Corporation may be attributed to the fact that they were defatted and also that the fresh liver was fed at a near maximum level. However, more samples must be tested before definite conclusions can be made. It can be seen from the results that drying the liver at 70° did not noticeably alter the vitamin B<sub>12</sub> content. The variations in pork samples obtained in this work and in previous work<sup>2</sup> are undoubtedly due to the fact that swine are monogastric animals and therefore do not benefit from the nutrients that may be produced by microorganisms in the rumen of cattle. The high level found in horse meat indicates that the synthesis of vitamin B<sub>12</sub> in the cecum of the horse may be as efficient as in the rumen of cows and sheep.

**Summary.** The vitamin B<sub>12</sub> content of a number of natural materials as determined by the rat assay is presented. Fish solubles, streptomycin "slops", sheep rumen contents and glandular meats are excellent sources of vitamin B<sub>12</sub>. Muscle tissue, eggs and milk

<sup>3</sup> Hall, B. E., Morgan, E. H., and Campbell, D. C., *Proc. Staff Meet., Mayo Clin.*, 1949, **24**, 99.

<sup>4</sup> Shive, W., Ravel, J. M., and Eakin, R. E., *J. Am. Chem. Soc.*, 1948, **70**, 2614.

<sup>5</sup> Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, 1948, **175**, 475.

<sup>6</sup> Short, M. S., *J. Bact.*, 1947, **53**, 669.

<sup>7</sup> Lewis, U. J., Register, U. D., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 509.

TABLE I.  
 Vitamin B<sub>12</sub> Content of Natural Materials.

Daily supplement	Avg growth 2 week assay period, g	Min. vit. B <sub>12</sub> content fresh basis, µg/100 g	Min. vit. B <sub>12</sub> content dry basis, µg/100g
None	35 ± 5		
0.1 µg vit. B <sub>12</sub> , inj.	66		
0.1 µg vit. B <sub>12</sub> , orally	65		
1 mg thymidine, inj.	36		*
2 mg thymidine, orally	36		*
250 µg thymine, uracil, adenine, guanine, and 500 µg rutin and hesperidin	35		*
10 ml tomato juice, fresh	34	*	
6% dried streptomycin "slop"	95		>22
6% condensed fish solubles	96	20	>40
5% herring stickwater	55	7	14
5% desiccated hog mucosa	43		trace
2% desiccated swine adrenals	57		15
1.25% desiccated sheep rumen contents	76		50
2.5% desiccated sheep rumen contents	93		50
10% crude casein	53		3
10% purified casein (vitamin test)	15		*
10 ml raw milk (cow)	41	trace	
20 ml raw skimmed milk (cow)	42	trace	
25 ml raw milk (goat)	28	*	
15% whole milk powder (Klim)	61		2.5
Rennet ppt. casein 25 g milk†	50	0.12	1
15% malted milk	54		2.2
2.5 g cheese cheddar	56	1.4	2.3
4 g egg yolk	64	1.4	2.8
1 g beef liver (fresh)	82	15	>50
1 g beef kidney (cooked)	90	20	>50
.125 g viobin liver powder, 37°C‡	80		100
.125 g viobin liver powder, 70°C‡	78		97
5 g beef round, sample 1 (cooked)	74	2	3.6
5 g beef round, sample 2 (cooked)	83	3	5.5
2.5 g beef round, sample 2 (cooked)	68	3	5.5
5 g beef tongue (cooked)	91	3	5.5
.75 g chicken liver (fresh)	70	11	35
5 g pork shoulder, sample 1	73	2	3.6
5 g pork shoulder, sample 2	67	1.2	2.2
5 g pork hocks	51	0.5	0.9
5 g pork ham	68	1.2	2.2
5 g mutton	82	3	5.5
5 g veal	76	2	3.6
5 g horsemeat (canned)	84	3.4	7.5

\* No measurable quantity.

† Calculated on fresh milk basis.

‡ Defatted and desiccated.

min B<sub>12</sub> it is possible to supply standard amounts to rats and thereby calculate the actual amount of vitamin B<sub>12</sub> present in the samples tested.

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† Register, U. D., Ruegamer, W. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1949, **177**, 129.

We wish to present in this paper a preliminary report of the distribution of vitamin B<sub>12</sub> in a variety of biological materials.

*Experimental and Results.* The assay procedure has been described in detail in a previous paper.<sup>1</sup> Male weanling rats are placed on a corn-soybean-basal ration containing 0.1% iodinated casein† for a 2 week depletion

<sup>2</sup> Register, U. D., Lewis, U. J., Thompson, H. T., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 167.

† Supplied by Dr. W. R. Graham, Cerophyl Laboratories, Inc., Kansas City, Mo.

period. At the end of this time supplements are administered for another 2-week period during which time the growth response is followed. The vitamin B<sub>12</sub> content of the sample is calculated through the use of a standard growth curve plotted from data obtained with graded levels of crystalline vitamin B<sub>12</sub>.<sup>2</sup>

Five rats were used for each assay and an average of the growth response for these rats was taken. In most cases the material to be tested was added on a percentage basis to the basal ration and the vitamin B<sub>12</sub> content was calculated from the total food consumed. In other cases, due to the nature of the material, the supplement was given in separate food containers and the amount consumed was measured directly. The results are summarized in Table I.

Average growth responses on the basal ration during the 2 week assay period have been found for a large number of animals to be 35 g ( $\pm$  5 g). The daily administration of 0.1  $\mu$ g of vitamin B<sub>12</sub> either orally or by injection, increased the growth response to 65-66 g. Since Hall, *et al.*<sup>3</sup> showed that incubation of vitamin B<sub>12</sub> with gastric juice increased the hematopoietic activity in pernicious anemia when administered orally, a similar treatment was tried for the rat. No effect on the activity of vitamin B<sub>12</sub> was noted after incubation with human gastric juice.

Thymidine has been found to be capable of replacing vitamin B<sub>12</sub> in the nutrition of *Lactobacillus lactis*.<sup>4,5</sup> Results presented in Table I show that this compound has no activity in the rat assay. Fresh tomato juice, which has been found to contain the TJ factor required by *Lactobacillus lactis* Dorner,<sup>6</sup> was likewise found to be inactive. Furthermore, a combination of thymine, uracil, adenine, guanine, rutin and hesperidin produced no growth response. It appears, therefore, that the rat

assay is rather specific for vitamin B<sub>12</sub>.

Two samples of yeast autolysates, U2 and Z7, supplied by Dr. C. N. Frey, Standard Brands, Inc., were found to be completely inactive. A sample of "slops" from streptomycin production was found to be very high in vitamin B<sub>12</sub>. The regular commercial condensed fish solubles showed high activity while a sample of herring stickwater was not as active.

Sheep rumen contents were very active, which suggests a synthesis of this vitamin within the rumen. Milk, cheese, meats and egg yolk were found to be relatively good sources of the vitamin. Dried soybean sprouts, barley, fresh potatoes, beans, cabbage, green peas, alfalfa leaf meal, and fresh grass contained no significant amount.

The vitamin B<sub>12</sub> content of beef kidney was found to be as high as that of beef liver. This is in agreement with previous results obtained on the vitamin B<sub>12</sub> content of liver and kidneys from rats fed different rations.<sup>7</sup> The higher values obtained with the two liver powder preparations of the VioBin Corporation may be attributed to the fact that they were defatted and also that the fresh liver was fed at a near maximum level. However, more samples must be tested before definite conclusions can be made. It can be seen from the results that drying the liver at 70° did not noticeably alter the vitamin B<sub>12</sub> content. The variations in pork samples obtained in this work and in previous work<sup>2</sup> are undoubtedly due to the fact that swine are monogastric animals and therefore do not benefit from the nutrients that may be produced by microorganisms in the rumen of cattle. The high level found in horse meat indicates that the synthesis of vitamin B<sub>12</sub> in the cecum of the horse may be as efficient as in the rumen of cows and sheep.

**Summary.** The vitamin B<sub>12</sub> content of a number of natural materials as determined by the rat assay is presented. Fish solubles, streptomycin "slops", sheep rumen contents and glandular meats are excellent sources of vitamin B<sub>12</sub>. Muscle tissue, eggs and milk

<sup>3</sup> Hall, B. E., Morgan, E. H., and Campbell, D. C., *Proc. Staff Meet., Mayo Clin.*, 1949, **24**, 99.

<sup>4</sup> Shive, W., Ravel, J. M., and Eakin, R. E., *J. Am. Chem. Soc.*, 1948, **70**, 2614.

<sup>5</sup> Wright, L. D., Skeggs, H. R., and Huff, J. W., *J. Biol. Chem.*, 1948, **173**, 475.

<sup>6</sup> Short, M. S., *J. Bact.*, 1947, **53**, 669.

<sup>7</sup> Lewis, U. J., Register, U. D., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 509.

products contain lesser amounts, whereas plant materials show no measurable activity.

We are indebted to Merck and Co., Inc., Rahway, N. J., for crystalline vitamins, and to Dr. B. L.

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## Observations on Diaphragm and Stomach of the Dog Following Phrenicotomy.\* (17476)

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In a recent communication we have pointed out some of the relationships between the left diaphragm and gastro-intestinal mechanisms in the dog.<sup>1</sup> The significant feature of these observations was a decrease in gastric emptying time in the presence of a relatively dilated stomach after left sided transthoracic phrenicotomy. The dogs were kept for prolonged periods in order to observe whether the denervated diaphragm would resume its respiratory function. This was done in view of Noack's work, who reported that regular rhythmic respiratory contractions of the diaphragm reappeared in the human from one and a half to two years after phrenic exeresis in the neck.<sup>2</sup> Noack's statement has been accepted by a number of authors. The mechanism of recovery of diaphragmatic function was postulated to be through the twelfth thoracic intercostal nerves and through sympathetic innervation. If the innervation of the diaphragm and recovery of its function following interruption of the phrenic nerve in man and dog are comparable, experiments on the latter offer the advantage of better control of most experimental conditions and ensure a complete section of the phrenic nerve, which is not always possible in the usual opera-

tions in the neck of man for phrenic exeresis. Long term observations comparable to those done by Noack on the human were done on the dog. The animals were observed fluoroscopically, in order to determine if and when there would occur reactivation of the diaphragm after complete left transthoracic phrenic section, the right diaphragm serving as normal control. Sauerbruch<sup>3</sup> described a gastrocadiac symptom complex following left side phrenic exeresis, which was later called Roemheld's syndrome. The symptoms were bradycardia, premature systoles, anxiety, epigastric distress, lack of appetite, pallor, nausea, vomiting and eructations.

*Experimental.* Sixteen dogs were operated upon transthoracically under aseptic conditions, using pentobarbital sodium anesthesia. In 13 animals a section of the left phrenic nerve from the hilum of the lung to the diaphragm was removed. Two dogs had simple phrenic section at the level of the diaphragm, and one had the nerve cut at the hilum of the lung. The cut ends of the nerve were doubled up and tied with non-absorbable suture. The dogs appeared normal after a few days of postoperative care. All dogs were fluoroscoped at weekly intervals for the first 12 to 14 weeks after operation and then at monthly intervals, to watch for possible reactivation of the diaphragm and for the possible development of Roemheld's syn-

\* Aided by a grant from the Otto Baer Fund.

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1 Jefferson, Nelson C., and Necheles, H., *Proc. Soc. Exp. Biol. and Med.*, 1948, **68**, 166.

2 Noack, R., *Beitr. Z. Klin. d. Tuberk.*, 1933, **82**, 397.

3 Quoted by Roemheld, L., *Munch. Med. Wchnschr.*, 1928, **75**, 1872.

drome. Observations were made also after standard barium meals<sup>4</sup> to determine if there was a relationship between the possible reactivation of the diaphragm and the return to normal of the gastric emptying time. Eight of the dogs were observed for periods of 14, 15, 16, 18, 19, 19, 20 and 20 months post-operatively, at which time acute experiments were done under pentobarbital sodium anesthesia and with artificial respiration, to determine whether impulses would reach the diaphragm upon stimulation of the phrenic nerve above the point of the previous section. In these experiments, gastric motility and blood pressure recordings were made before and after stimulation of the phrenic nerve with various strengths of induced current from a Harvard inductorium. At the termination of all experiments sections were made from the right and left diaphragm and from the proximal and distal phrenic nerve stumps for microscopic study.

**Results.** Using fluoroscopy, we have not seen in any of 15 dogs a completely denervated diaphragm reactivate during 20 months of observations. The shortened gastric emptying time returned to normal in periods varying from several weeks to several months. The large gas bubble within a moderately dilated stomach however persisted for many months. At no time was respiratory difficulty observed, but the dogs were not subjected to severe exercise. Acute experiments on 8 dogs 14, 15, 16, 18, 19, 19, 20 and 20 months after phrenic section revealed a fixed, elevated, atrophied left diaphragm more fixed by intra-abdominal pressure than by adhesions. The muscle was paper-thin and translucent. In one dog (19 months) there was a completely atrophied antero-lateral one half of the left diaphragm and a normal postero-lateral half. Upon inspection of the opened chest under anesthesia, the normal part showed rhythmic contractions, while the atrophied part did not. However careful dissection and inspection revealed one intact accessory phrenic nerve, which had not been cut. In this animal, fluoroscopy had been difficult, and we were

not certain whether the diaphragm moved or not.

Sections<sup>†</sup> from the left diaphragm revealed degeneration and disappearance of nerve fibers and degenerated muscle, replaced by fibrous tissue and fat. Control sections from the right diaphragm were normal. The phrenic nerve above the point of section revealed degenerating, regenerating, or normal nerve fibers. The phrenic nerve distal to the section revealed complete degeneration. Stimulation of the distal or proximal ends of the cut phrenic nerve did not reveal any effect on the diaphragm in the 7 dogs with complete phrenicotomy, which were tested in acute experiments. Stimulation of the proximal phrenic stump caused a rise in blood pressure and, in some experiments, increased respiration. When the stump was cut central to the electrode these reflexes were abolished. The gastro-cardiac symptom complex of Roemheld was never observed.

**Discussion.** Credit for first interrupting the phrenic nerve in man is given to Stuerz<sup>5</sup> and to Sauerbruch.<sup>6</sup> The exhaustive study of Felix<sup>7</sup> established a basis for the operation. He found that the phrenic nerve receives sympathetic fibers in the neck and at the level of the diaphragm, as well as spinal fibers from lower cervical and first thoracic nerves. The phrenic does not give branches to the pericardium. The left phrenic divides into an anterior, lateral and posterior branch to supply the diaphragm. The importance of an accessory phrenic is stressed, which may be easily missed by the surgeon, and which will allow for continued phrenic impulses to the diaphragm. He concludes that the motor innervation of the diaphragm consists of the phrenic, the 12th intercostal nerve, and probably sympathetic fibers. The phrenic supplies the main part of the diaphragm, the 12th intercostal the root of the diaphragm from the 12th rib, and sympathetic and phrenic to-

<sup>†</sup> We acknowledge the help of Dr. O. Saphir, Pathologist of Michael Reese Hospital.

<sup>5</sup> Stuerz, *Deutsche Med. Wchnschr.*, 1911, **37**, 2224, quoted by Felix.<sup>7</sup>

<sup>6</sup> Sauerbruch, F., *Chirurgie der Brustorgane*, ed. 3, II, Julius Springer, Berlin, 1930.

<sup>7</sup> Felix, W., *Deutsche Z. f. Chir.*, 1922, **171**, 283.

<sup>4</sup> Kozell, D. D., and Neeheles, H., *Surg.*, 1942, **2**, 360.



gether supply the lumbar section of the diaphragm. Schwatt<sup>8</sup> studied 138 cases after phrenic exeresis or/and phrenicotomy and observed that, during the earlier period of from two to eighteen months after the operation, paradoxical movements of the diaphragm were the rule. Ultimately however, probably because of degeneration and atrophy of the diaphragmatic musculature plus pleural adhesions, fixation occurred. Davies<sup>9</sup> lists 3 operations for paralyzing the diaphragm, namely: phrenic avulsion, phrenicotomy, and phrenic crush. "The immediate effect of these operations is paralysis of the hemidiaphragm; the muscle fibers degenerate and the dome becomes a sheet of fibrous tissue within approximately four months. As a result of the paralysis there are three changes of the dome, namely: a loss of tone, a rise into the thorax, and a cessation of respiratory movements." Klein<sup>10</sup> states that many failures to secure paralysis of the hemidiaphragm are due to the presence of accessory branches of the phrenic nerve, which join the main trunk deep in the thorax, and which can carry on the function of the phrenic nerve if they are not cut. Twenty per cent to thirty per cent of all individuals are supposed to have accessory branches. Geraghty and Aycock<sup>11</sup> state that the length of phrenic nerve avulsed is of importance, because of the possibility of return of diaphragmatic function unless all auxiliary branches are interrupted. In their series the maximum avulsed length was 30 cm., and the minimum 2.5 cm. Matson<sup>12</sup> states that an avulsion of not less than 10 cm means a return of diaphragmatic function in 25 to 30% of all patients. Schwatt<sup>13</sup> concludes that an avulsion of 10 to 12 cm is necessary, as this approximates the distance from the operative site to the hilum and was sufficient to interrupt all accessory fibers. Anderson<sup>14</sup>

states that the diaphragm may continue to rise for 6 months and that the immediate rise is by no means the maximum. Aufses,<sup>15</sup> in a review of the anatomy of the phrenic nerves, states that most of the accessories are not too intimately connected with blood vessels and join the main trunk within a distance of 11 to 13 cm from the level at which it crosses the scalenus anticus. Therefore, if avulsion of the nerve is performed, and at least 13 cm are removed, one may be confident that a total permanent paralysis of the hemidiaphragm will ensue. Further he states that the phrenic nerve is the sole motor nerve to its corresponding hemidiaphragm, and that interruption causes immediate paralysis of the muscle with atrophy which begins in a few weeks and progresses until it becomes complete at about the third or fourth month.

Aree<sup>16</sup> states that the phrenic nerve has anastomoses with the sympathetic system when it enters the thorax, and that some of the branches of the phrenic are distributed to the pulmonary hilum. The latter fact makes it possible that phrenic section may produce a decrease of pulmonary reflexes. Textbook information assures that in the human, following crushing of the phrenic nerve, the diaphragm reactivates in about 3 months.<sup>17</sup> According to Noack,<sup>2</sup> after exeresis in the neck of 10 to 30 cm of phrenic nerve, the diaphragm reactivated in from 1½ to 2 years, and he attributes this to fibers of the twelfth intercostal nerve and to sympathetic innervation. Christopher<sup>17</sup> states that for permanent paralysis not less than 4½ to 5 inches of the main trunk of the phrenic nerve must be avulsed from within the thorax. This will usually sever accessory branches (present in 20% of cases) which join the main nerve along its course to the diaphragm. Occasionally one or more accessory nerves may have an independent course to the diaphragm. Therefore avulsion of the main nerve may or may not interrupt all accessory branches of the phrenic nerve. Since after 20

<sup>8</sup> Schwatt, H., *Am. J. M. Sc.*, 1934, **187**, 338.

<sup>9</sup> Davies, H. M., *The Lancet*, 1935, **229**, 418.

<sup>10</sup> Klein, B., *Illinois Med. J.*, 1938, **73**, 418.

<sup>11</sup> Geraghty, F. J., and Aycock, T. B., *The West Virginia Med. J.*, 1940, **36**, 546.

<sup>12</sup> Matson, R. W., *Am. Rev. Tuberc.*, 1930, **22**, 1.

<sup>13</sup> Schwatt, H., *J. Thor. Surg.*, 1934, **3**, 503.

<sup>14</sup> Anderson, B. W., *Edinburgh Med. J.*, 1934, **41**, 169.

<sup>15</sup> Aufses, A. H., *Am. J. Surg.*, 1940, **50**, 715.

<sup>16</sup> Aree, J., and Breu, M. M., *J. Thor. Surg.*, 1943, **12**, 544.

<sup>17</sup> Christopher, F., *A Textbook of Surgery*, 3rd ed., W. B. Saunders Co., Philadelphia and London, 1943.

months we have found only atrophy of the denervated diaphragm in our dogs it would appear that either the innervation is not the same in the dog as in the human, or that our procedure on the dog involved complete section, while in man, where the procedure is done in the neck, all phrenic fibers may or may not be avulsed, no matter how much of the main nerve is removed. No emphasis seems to be placed by the many writers on tying the cut ends of the nerve with non-absorbable suture, a feature which to us appears to be of some importance. We have found that the phrenic nerve is able to regenerate within a period of 20 months.<sup>‡</sup> Since the musculature of the diaphragm in both species consists of striated muscle, and the phrenic is a somatic nerve, it follows that, once true paralysis and atrophy has developed in the muscle it would never be capable of reactivation. The so-called paralysis as seen in the human is probably in many instances palsy and not paralysis, as outlined by Christopher.<sup>17</sup>

The fact that we obtained complete paralysis and not palsy may be speculated upon. Our operations were transthoracic, and all observable fibers were cut and tied with a non-absorbable suture. The one instance observed by us in which partial diaphragmatic function persisted and in which complete atrophy of one half of the left diaphragm was found 19 months after supposed complete section of the phrenic nerve, was demonstrated to be due to an intact accessory phrenic nerve missed at operation. This would of course indicate that once atrophy, always atrophy, and that in the human so-called reactivation might have been confused with such a picture as we observed in this animal.

The reason for the absence of Roemheld's syndrome in our dogs may lie in the expla-

nation given by Noack,<sup>2</sup> namely, that the tearing of sympathetic fibers during phrenic avulsion in the neck and not section of the phrenic nerve itself leads to the syndrome.

Paradoxical movements of the diaphragm following phrenicotomy, as described by Schwatt<sup>8</sup> were not observed in our experiments.

*Summary.* 1. No reactivation of the left diaphragm was seen by us in 15 dogs up to 20 months after complete section of the left phrenic nerve.

2. Microscopically all diaphragms showed complete muscular atrophy with degeneration or disappearance of nerve fibers, 14 to 20 months after complete section of the left phrenic nerve. It is difficult to conceive how such atrophic muscles could ever regenerate.

3. It is highly possible that phrenic exeresis in the neck of the human does not always result in complete phrenicotomy with subsequent atrophy of the diaphragm, but in a number of instances results in diaphragmatic palsy.

4. We found no evidence that the twelfth thoracic intercostal nerve or sympathetic innervation could influence the reactivation of the diaphragm in the dog.

5. Our experience with one diaphragm leads us to believe that a hemifunction of one half of a diaphragm may lead the fluoroscopist to assume that complete reactivation of that diaphragm has occurred.

6. The phrenic nerve above the point of section was able to convey reflexes 20 months after section; this was demonstrated by rise in blood pressure and increased respiration upon stimulation of the central end of the nerve, which were abolished when the nerve was cut central to the stimulating electrode.

7. No evidence of the gastro-cardiac symptom-complex of Roemheld was seen.

<sup>‡</sup> Unpublished work.

# The Coenzyme A Content of Ischemic Dog Myocardium. (17477)

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We have previously reported the destruction of coenzyme I in dog myocardium rendered ischemic by ligation of the left anterior descending coronary artery. In similar experiments cocarboxylase was found unchanged after ischemia.<sup>1</sup> In the case of coenzyme I, the destruction described could be prevented by pretreatment with large doses of nicotinamide.

It may be considered that the occurrence of breakdown of a coenzyme under these conditions can suggest the likelihood of favorable results in treatment of coronary occlusion with large doses of the vitamin corresponding to the coenzyme concerned. On this basis it was thought of interest to determine whether or not coenzyme A is broken down in ischemic myocardium and whether the breakdown, if any, may be prevented by pantothenic acid administration.

**Methods.** The left anterior descending coronary artery was ligated in ten dogs by essentially the same technique reported previously. Ischemic and control myocardial samples were removed after 2 hours, boiled, homogenized, and analyzed for coenzyme A by the method of Kaplan and Lipmann.<sup>2</sup>

Four of the 10 dogs were pretreated with 50 mg/kg of sodium pantothenate intravenously 10 minutes before ligation of the vessel.

**Results.** The accompanying table shows in Kaplan-Lipmann units per gram dry muscle, the myocardial content of coenzyme A together with the percent change after 2 hours of ischemia. It is evident that breakdown of coenzyme A does occur, although by no means to the extent that coenzyme I is degraded, and that pantothenate does not significantly relieve the loss of coenzyme. This could suggest that the breakdown is not enzymically catalyzed, that heart muscle does not synthesize coenzyme A, or that pantothenic acid needs molecular rearrangement before being available for coenzyme A synthesis by myocardium.

**Summary.** Acute coronary artery ligation in the dog results in loss of coenzyme A in the infarcted myocardial area. Pretreatment with pantothenic acid does not influence this loss.

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TABLE I.  
Myocardial Coenzyme A Content in Lipmann Units/G Dry Wt.

Not pretreated				Pretreated			
Dog	Normal	Ischemic	% change	Dog	Normal	Ischemic	% change
A	166	53	-68	G	227	111	-53
B	173	106	-39	H	184	162	-12
C	204	155	-24	J	168	143	-15
D	166	145	-13	K	125	107	-14
E	176	155	-12				
F	145	90	-38				

<sup>1</sup> Govier, Wm. M., *Am. Heart J.*, 1945, 29, 384.

<sup>2</sup> Kaplan, Nathan O., and Lipmann, Fritz, *J. Biol. Chem.*, 1948, 174, 37.

## Effect of Protein Depletion upon Susceptibility of Rats to Total Body Irradiation. (17478)

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Although there have been many studies of the effects of penetrating radiation on normal animals of various species, there has been little investigation of the effects of radiation upon abnormal animals. Some components of diet, particularly the vitamin B series<sup>1</sup> and some of the amino acids,<sup>2</sup> have been reported to alter the response to radiation but the broader problems of protein, fat, or carbohydrate nutrition have not, to our knowledge, been investigated to any great extent. Since widespread tissue necrosis is a prominent feature of radiation damage, it appeared that investigation of the effect of protein, particularly a deficiency of protein, on the response to radiant energy might prove instructive. Therefore a series of experiments, of which this is the first report, was undertaken to try to evaluate the role of protein in radiation damage.

**Procedure.** This experiment was planned to determine the effect of moderately severe depletion of the protein reserves on the susceptibility to radiation. Since young adult rats showing 25% reduction in weight while on a protein-poor diet are known to have but little reserve protein,<sup>3</sup> this type of animal was selected for this study. There is considerable confusion regarding the effect of age and weight on susceptibility to radiation. For this reason two types of control animals were used, one group having the same weight as the test animals, the other being the same age. A total of 141 rats was almost equally divided into the 3 groups in this experiment. Protein-depletion, as it is

produced by the method developed by Cannon and his group, consists of placing the rats on a low protein diet that is adequate in calories, vitamins, and minerals. The diets and methods used in obtaining protein-depleted rats have been described elsewhere by Cannon<sup>4</sup> and Wissler.<sup>5</sup> In this experiment male albino rats of the Sprague-Dawley strain were used. Rats with an initial weight of about 200 g were placed on a diet (3E) containing 1.9% protein. The animals were kept in large, wire-bottom cages with 6 rats to a cage. The rats were continued on the depletion diet until they had lost 25% of their starting weight. Such a weight loss occurred in about 10 weeks. The animals were then given total body irradiation.

Total body irradiation of all of the animals was accomplished by x-ray. The radiation was done at 200 kv and 15 ma with 0.5 mm copper and 3 mm bakelite filters. The half-value layer of the radiation was 1.2 mm of copper. The target to animals distance was 71 cm and the dose rate in all cases was 17-18 r per minute. The dosage rate was measured before and after each exposure with a Victoreen electrometer. The animals were placed in individual perforated aluminum cages and radiated in a horizontal beam. The cages were regrouped and turned several times during the exposure to give the animals uniform distribution in the beam.

The two groups of control rats were maintained on a diet (3C) identical with the protein-deficient ration except that it contained 22% protein supplied by casein. The age control rats were placed on this diet after they had reached a starting weight of about 200 grams. The animals were continued on this program until they had been

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<sup>1</sup> Martin, C. L., Moursand, W. H., *Radiology*, 1938, 30, 277.

<sup>2</sup> Patt, H. M., Tyree, E. B., Straube, R. L., Smith, D. E., *Science*, 1949, 110, 213.

<sup>3</sup> Benditt, E. P., *Proc. Soc. Exp. Biol. and Med.*, 1949, 70, 243.

<sup>4</sup> Cannon, P. R., Wissler, R. W., Woolridge, R. L., and Benditt, E. P., *Ann. Surg.*, 1944, 120, 514.

<sup>5</sup> Wissler, R. W., Woolridge, R. L., Steffee, H. S., and Cannon, P. R., *J. Immun.*, 1946, 52, 267.

on the 22% protein diet for a period of time equal to the period of depletion for the protein-depleted rats. At time of radiation these animals were 20 weeks old and weighed about 280 g. For the second control group, rats with an initial weight of about 100 g were fed the 22% protein ration at a rate of 15 g per rat per day until they had reached a weight of approximately 150 g. These rats were then about 8 weeks old and were given total body irradiation. Three groups of these rats were radiated before they had reached a weight of 150 g. The results show that this did not lessen their value as control animals.

During the 30-day observation period following radiation all of the animals were continued on the same diets they had been receiving prior to radiation. All of the rats were weighed at 2-day intervals through this observation period.

These experiments were performed over a 9-months period. For the most part corresponding groups of depleted and age control animals were radiated at the same time and these groups were under observation at the same time with similar animal colony conditions. It was not feasible to radiate weight control animals at the same time as the other groups. Nevertheless groups of all 3 types of rats were scattered through the 9-months period in an attempt to keep the conditions in each series as nearly comparable as possible.

**Results.** The results of this experiment are shown in Table I. Here it is apparent that the mortality in the protein-depleted animals was considerably greater at any given dosage than in the two control groups. Most of the deaths in the protein-depleted groups of rats occurred below 625 r while there were few deaths in the control groups below this dose.

In order to plot definite dosage-mortality relationships, the data were treated in the manner developed by Bliss.<sup>6,7</sup> The regression lines derived from this procedure are shown in Fig. 1. This treatment of the data demonstrates clearly that the protein-de-

TABLE I.  
Mortality in Rats Following Total Body Irradiation.

Dose	No. animals	Avg wt, g	Died
Depleted rats			
300r	4	158	1
450	6	160	0
525	6	162	1
525	3	154	2
575	6	146	2
575	3	155	3
600	6	156	6
625	3	150	2
625	3	161	3
750	6	157	5
Age controls			
525r	3	286	0
575	6	293	0
625	3	304	0
650	3	297	1
675	9	268	6
700	3	299	1
725	9	269	6
750	3	299	2
775	9	269	8
800	2	291	2
Wt controls			
500r	5	155	0
600	5	153	2
600	10	128	1
650	10	128	3
700	5	156	4
700	10	128	4

pleted rats were considerably more susceptible to total-body irradiation than were either animals of the same age or of the same weight when fed adequate protein in their diet.

The equation for the regression line for the protein-depleted animals is:  $Y = 5.3460 + 8.9179 (X - 2.7463)$  where  $Y$  equals the probit value of the mortality and  $X$  is the logarithm of the observed dose. The standard deviation for this line is .1121. The regression line of the animals of the same age is:  $Y = 5.1545 + 21.0772 (X - 2.8476)$  with a standard deviation of .0474. Rats of the same weight give:  $Y = 4.7268 + 15.0372 (X - 2.8135)$  and a standard deviation of .0665. The fiducial ranges, or confidence limits of the dosage, with a probability of .05 for the regression lines at the lethal dose for 50% of the animals in 30 days (LD 50/30) were calculated according to Bliss<sup>6</sup>

<sup>6</sup> Bliss, C. I., *Ann. Applied Biol.*, 1935, **22**, 134.

<sup>7</sup> *Ibid.*, 1935, **22**, 307.

<sup>8</sup> Bliss, C. I., *Quart. J. and Yearbook Pharmac.*, 1938, **11**, 192.

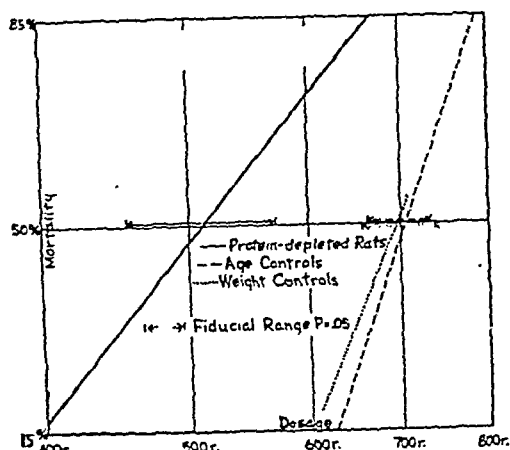


FIG. 1.

Thirty-day dosage-mortality regression lines in rats following total body irradiation.

and are indicated by corresponding arrows in Fig. 1. As can be seen, there is a wide zone separating the limits of variation of the protein-depleted animals from the limits of the controls.

In this experiment the weights of the protein-depleted radiated rats that survived the 30-day observation period closely paralleled the weight loss in non-radiated depleted animals except for some additional weight loss in the period immediately following radiation. Both the age control and the weight control rats also showed weight loss in the first few days following radiation when diet consumption was poor. This loss was quickly recovered when these animals regained their appetite and they continued to gain weight through the remainder of the observation period. It is important to note that a period of 100 days on the protein-deficient diet, as was the case with these depleted animals, causes no mortality in non-radiated rats. Rats have been kept on a protein-deficient diet for as long as 6 months in this laboratory with little spontaneous mortality, providing care is taken to prevent any infectious diseases of rats. Thus it is not likely that any of the increased mortality observed in the protein-depleted rats was due to spontaneous mortality.

In all groups of radiated animals, as is the case in most radiation mortality studies,

death usually occurred before the fifteenth post-irradiation day. Autopsies were done on most of the rats that died and no differences were noted between the lesions in the control and depleted rats. Most of the animals that died in the first few days following radiation showed widespread interstitial hemorrhages. Some of the rats that died later showed pneumonia. Blood cultures were done on 12 of the protein-depleted rats that died after exposure of less than 650r. Two organisms were found consistently in the 10 animals that died before the fifteenth post-irradiation day. One organism was a gram-negative rod (probably *Bacillus dysenteriac*) and the other a micrococcus. The 2 depleted animals that died after the fifteenth day both had sterile blood cultures.

**Discussion.** The present experiment shows that the protein-depleted rat is materially more susceptible to the physical injury of penetrating radiation than is the animal on an adequate protein diet. The LD 50/30 for the protein-depleted rat is approximately 520r while the LD 50/30 for the control rats in this series proved to be in the region of 700r. Thus at the LD 50/30 there is a great difference in the dosage between depleted and control rats. This increased susceptibility of the protein-depleted rat becomes even more apparent when one realizes that the dose of total body irradiation that killed 50% of the protein-depleted rats killed less than two per cent of the control rats. The slope of the regression line for the protein-depleted animals indicates that these animals are proportionately somewhat more susceptible to lower doses of radiation than the controls.

The fiducial range for each of the regression lines, as shown in Fig. 1, shows a wide zone separating these ranges at the LD 50/30. Calculation of the differences at the LD 50/30 indicates that the P value for the data at this point is somewhat less than .001. Therefore the observed increase in mortality from radiation in the protein-depleted rat is highly significant.

Since there is confusion about the influence of weight and age on radiation suscepti-

bility, control rats for both of these factors were used. Quastler<sup>9</sup> found that in mice, with doses in the range of the LD 50, the survival time was proportional to body weight and age. Hagen and Sacher<sup>10</sup> and Hagen and co-workers<sup>11</sup> reported that in a series of mortality studies on rabbits the animals weighing more than 2 kg were less sensitive to x-ray than rabbits weighing less than 2 kg. Naiman<sup>12</sup> recently reported that older, and therefore heavier, rats could sustain larger doses of radiation than small rats. In contrast to these conclusions Hagen and Simmons<sup>13</sup> studied a series of 638 rats of the same strain as that used in this experiment and concluded that "within the range tested (median weight 210 g) any age or weight effect on radioresistance of rats must be small." Boche and Bishop in a study of 240 rats of the Wistar strain concluded that age and sex did not cause significant differences in radiation mortality.<sup>14</sup> The coincidence of the two control regression lines in this experiment and the corresponding fiducial ranges indicate that age and weight in the adult rat, in the range of 150 to 280 g, have little effect on susceptibility to radiation under the conditions of this study.

The LD 50/30 of about 700r for the 2 groups of control rats in this experiment is in close agreement with the LD 50/30 being obtained at present for similar animals in another laboratory.<sup>15</sup> This figure is in some disagreement with a LD 50/30 of 600r as reported by Prosser<sup>16</sup> but is in only moderate disagreement with a LD 50/30 of  $640 \pm 5r$  for a Wistar strain of rats as recently reported by Clark and Uncapher.<sup>17</sup>

The finding of increased susceptibility to radiation damage in protein-depletion coincides with findings of increased susceptibility in protein-depleted rats to other types of injury. The studies of Cannon,<sup>4,18</sup> Wissler<sup>7,19</sup> and Benditt<sup>3</sup> have demonstrated that antibody production is seriously impaired by severe and prolonged protein-depletion. Taliaferro and associates<sup>20</sup> recently demonstrated a decreased immunity to trichinosis in the protein-depleted rat. A delay in wound healing was noted by Kobak and associates<sup>21</sup> in protein-depletion. The leukocytic response of the bone marrow, particularly to repeated stimulation, is also markedly impaired by depletion of protein reserves.<sup>22</sup>

Protein-depletion, in addition to causing some decrease in plasma proteins and hemoglobin level,<sup>23</sup> also causes a decrease in production of several of the enzymes. Tobin and co-workers<sup>24</sup> have shown a reduction in hyaluronidase and antihyaluronidase. Benditt and associates<sup>25</sup> have demonstrated that cytochrome oxidase and succinoxidase are decreased in liver homogenates in protein-depletion. Kidney phosphatase is also reduced while the total liver phosphatase remains constant during the depletion of the protein reserves. It is known that radiation also inhibits several of the enzymes, particularly the sulfhydryl-containing enzymes, as shown by the recent work of Barron and his group.<sup>26</sup>

<sup>17</sup> Clark, W. G., and Uncapher, R. P., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 214.

<sup>18</sup> Cannon, P. R., Chase, W. E., and Wissler, R. W., *J. Immun.*, 1943, **47**, 133.

<sup>19</sup> Wissler, R. W., *J. Infect. Dis.*, 1947, **80**, 264.

<sup>20</sup> Taliaferro, W. H., Woolridge, R. L., and Benditt, E. P., *Science*, 1949, **109**, 443.

<sup>21</sup> Kobak, M. W., Benditt, E. P., Wissler, R. W., and Steffee, H. S., *Surg. Gyn. Ob.*, 1947, **85**, 751.

<sup>22</sup> Asirvatham, M., *J. Infect. Dis.*, 1948, **83**, 57.

<sup>23</sup> Benditt, E. P., Straube, R. L., and Humphreys, E. M., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 189.

<sup>24</sup> Tobin, J. R., Bergenstahl, D., and Steffee, H. S., *Arch. Biochem.*, 1948, **16**, 373.

<sup>25</sup> Benditt, E. P., Steffee, H. S., Hill, T., and Johnston, T. C., *Fed. Proc.*, 1949, **8**, 350.

<sup>26</sup> Barron, E. S. G., Dickman, S., Muntz, J. A., and Singer, S. P., *J. Gen. Physiol.*, 1949, **32**, 537.

<sup>9</sup> Quastler, H., *Am. J. Roentgen.*, 1945, **54**, 457.

<sup>10</sup> Hagen, C., and Sacher, G., *MDDC-1252* (CH-3754), 1946.

<sup>11</sup> Hagen, C., Jacobson, L. O., Murray, R., and Lear, P., *MDDC-999* (CH-2147), 1944.

<sup>12</sup> Naiman, D. N., *Am. J. Roentgen.*, 1949, **61**, 95.

<sup>13</sup> Hagen, C., and Simmons, E., *MDDC-1210* (CH-3815), 1945.

<sup>14</sup> Boche, R. G., and Bishop, F. W., *MDDC-250*, 1947.

<sup>15</sup> Patt, H. M., personal communication.

<sup>16</sup> Prosser, C. L., *Radiology*, 1947, **49**, 299.

The manner in which protein-depletion increases susceptibility to radiation is not known. Several methods by which radiation may cause death have been postulated and protein-depletion affects most of these mechanisms. Death following radiation may be due to infection subsequent to the break-down of the normal immunological processes. It is well known that protein-depletion interferes with immunological responses and this in combination with the effects of radiation on the epithelial barriers, particularly the intestinal epithelium, may account for the increased susceptibility. Also it may be more difficult for the depleted animals to recover from the leukopenia of radiation so that the phagocytic barrier against infection is lowered. There is much evidence that the degree and duration of leukopenia following radiation have great influence on mortality.<sup>27</sup>

Since it is known that protein-depletion and radiation both interfere with enzyme production or action, it may be that there is an additive effect that interferes with enzyme activity to account for the increased radiation susceptibility.

It may be that death is due to anemia following bone marrow damage. That this mechanism accounts for the increased susceptibility does not seem likely for protein-

depletion *per se* does not cause a marked lowering in the erythrocyte or hemoglobin levels. However it may interfere with the recovery of the bone marrow and with the production of both erythrocytes and leukocytes.

It seems possible that the increased susceptibility to radiation observed in these protein-depleted animals might be due to their poor immunological and leukocytic responses or to their decreased production of some essential enzyme. This problem is being investigated further.

**Conclusions.** 1. Protein-depletion in young adult male rats, as produced by a weight loss of 25% on a protein-poor diet, materially increases susceptibility to total body irradiation. 2. Age or weight in normally nourished adult rats, in the 150 to 280 g weight range, have little effect on susceptibility to radiation.

The author wishes to express his appreciation for advice and assistance to the following men: P. R. Cannon, R. W. Wissler, and E. P. Benditt, Department of Pathology, University of Chicago; A. M. Brues, Biology Division, Argonne National Laboratory, Chicago; L. J. Savage, Department of Mathematics, University of Chicago; and H. D. Landahl, Committee on Mathematical Biology, University of Chicago.

The animals used in this experiment were irradiated through the collaboration of the Biology Division, Argonne National Laboratory Chicago.

<sup>27</sup> Brues, A. M., and Rietz, L., ANL-4227, **183**, 1948.

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## An Autohemolytic Agent in Fetal Liver Extracts.\* (17479)

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In the course of studies dealing with enzymatic activity during fetal and extra-uterine development it was noted that extracts of fetal liver lysed the red cells of the same species. It was of interest to determine the nature of this phenomenon and to com-

pare the activity of adult and fetal extracts.

**Methods.** Thirty experiments were performed, mostly on pregnant guinea pigs and their fetuses. A few experiments were made with pregnant rats or newborn rats and their mothers. The pregnant animals were killed by decapitation and the maternal blood was collected in oxalated tubes. The fetuses were then quickly removed and decapitated and

\* This investigation was supported in part by a grant from the National Cancer Institute, U. S. Public Health Service.



bility, control rats for both of these factors were used. Quastler<sup>9</sup> found that in mice, with doses in the range of the LD 50, the survival time was proportional to body weight and age. Hagen and Sacher<sup>10</sup> and Hagen and co-workers<sup>11</sup> reported that in a series of mortality studies on rabbits the animals weighing more than 2 kg were less sensitive to x-ray than rabbits weighing less than 2 kg. Naiman<sup>12</sup> recently reported that older, and therefore heavier, rats could sustain larger doses of radiation than small rats. In contrast to these conclusions Hagen and Simmons<sup>13</sup> studied a series of 638 rats of the same strain as that used in this experiment and concluded that "within the range tested (median weight 210 g) any age or weight effect on radioresistance of rats must be small." Boche and Bishop in a study of 240 rats of the Wistar strain concluded that age and sex did not cause significant differences in radiation mortality.<sup>14</sup> The coincidence of the two control regression lines in this experiment and the corresponding fiducial ranges indicate that age and weight in the adult rat, in the range of 150 to 280 g, have little effect on susceptibility to radiation under the conditions of this study.

The LD 50/30 of about 700r for the 2 groups of control rats in this experiment is in close agreement with the LD 50/30 being obtained at present for similar animals in another laboratory.<sup>15</sup> This figure is in some disagreement with a LD 50/30 of 600r as reported by Prosser<sup>16</sup> but is in only moderate disagreement with a LD 50/30 of  $640 \pm 5r$  for a Wistar strain of rats as recently reported by Clark and Uncapher.<sup>17</sup>

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<sup>17</sup> Clark, W. G., and Uncapher, R. P., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 214.

<sup>18</sup> Cannon, P. R., Chase, W. E., and Wissler, R. W., *J. Immun.*, 1943, **47**, 133.

<sup>19</sup> Wissler, R. W., *J. Infect. Dis.*, 1947, **80**, 264.

<sup>20</sup> Taliaferro, W. H., Woolridge, R. L., and Benditt, E. P., *Science*, 1949, **109**, 443.

<sup>21</sup> Kobak, M. W., Benditt, E. P., Wissler, R. W., and Steffee, H. S., *Surg. Gyn. Ob.*, 1947, **85**, 751.

<sup>22</sup> Asirvatham, M., *J. Infect. Dis.*, 1948, **83**, 87.

<sup>23</sup> Benditt, E. P., Straube, R. L., and Humphreys, E. M., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 189.

<sup>24</sup> Tobin, J. R., Bergenstahl, D., and Steffee, H. S., *Arch. Biochem.*, 1948, **16**, 373.

<sup>25</sup> Benditt, E. P., Steffee, H. S., Hill, T., and Johnston, T. C., *Fed. Proc.*, 1949, **8**, 350.

<sup>26</sup> Barron, E. S. G., Diekmann, S., Muntz, J. A., and Singer, S. P., *J. Gen. Physiol.*, 1949, **32**, 537.

<sup>9</sup> Quastler, H., *Am. J. Roentgen.*, 1945, **54**, 457.

<sup>10</sup> Hagen, C., and Sacher, G., MDDC-1252 (CH-3754), 1946.

<sup>11</sup> Hagen, C., Jacobson, L. O., Murray, R., and Lear, P., MDDC-999 (CH-2147), 1944.

<sup>12</sup> Naiman, D. N., *Am. J. Roentgen.*, 1949, **61**, 95.

<sup>13</sup> Hagen, C., and Simmons, E., MDDC-1210 (CH-3815), 1945.

<sup>14</sup> Boche, R. G., and Bishop, F. W., MDDC-250, 1947.

<sup>15</sup> Patt, H. M., personal communication.

<sup>16</sup> Prosser, C. L., *Radiology*, 1947, **49**, 299.

TABLE I.  
 Hemolytic Action of Saline Extracts of Fetal and Maternal Guinea Pig Livers.

		Dilution (see text)			
		15.0	7.5	3.8	1.5
A. Against maternal red cells					
1. Unheated					
Fetal		++++	++++	++	±
Maternal		+	±	0	0
2. Boiled 1 min.					
Fetal		0	0	0	0
Maternal		0	0	0	0
3. Ppt. of boiled extr.					
Fetal		+	±	0	0
Maternal		0	0	0	0
4. Heated to 56°C 1 hr					
Fetal		++	++	0	0
Maternal		0	0	0	0
5. Effect of 0.5 cc serum (See text)					
Fetal		+++	++	±	0
Maternal		0	0	0	0
		20.0	10.0	5.0	2.5
B. Against fetal red cells					
Fetal		++++	+++	++	0
Maternal		±	0	0	0
		15.0	7.5	3.8	1.5
C. Against rat red cells					
Fetal		++++	+++	++	0
Maternal		0	0	0	0

If the extracts alone are allowed to stand for 12 hours and then tested, a marked increase in their ability to produce lysis is found. On the other hand, the addition of the sodium salt of moniodoacetic acid to the extracts in a final concentration of 0.003 M inhibits the increase of activity due to standing.

**Discussion.** The results indicate that saline extracts of fetal guinea pig liver possess greater lytic activity than similarly prepared adult (maternal) extracts. The activity is due to both heat-labile and heat-resistant factors but the heat-sensitive agent accounts for the greater part of the lysis under the conditions of these experiments. At present, we cannot account for the fetal-maternal difference reported here. There is, however, a considerable body of literature that may give us insight into the probable nature of some of the lytic agents or systems involved.

There is substantial support for the idea that at least 3 hemolytic systems involving lysins exist *in vivo*: (1) It is well known that the spleen plays an important role in preparing red cells for destruction. Rous,<sup>1</sup> Krumbhaar,<sup>2</sup> Dameshek,<sup>3,4</sup> and Ponder<sup>5</sup> have reviewed the evidence bearing on this. It has been suggested that the agent responsible for this action of the spleen is produced by enzymatic action and the resulting substance has been identified as lysolecithin.<sup>6,7,8</sup> This

<sup>1</sup> Rous, P., *Physiol. Rev.*, 1923, 3, 75.

<sup>2</sup> Krumbhaar, E. B., *Physiol. Rev.*, 1926, 6, 160.

<sup>3</sup> Dameshek, W., *New England Med. J.*, 1941, 224, 727.

<sup>4</sup> Dameshek, W., *New England Med. J.*, 1942, 226, 339.

<sup>5</sup> Ponder, E., *Hemolysis and Related Phenomena*, Grune and Stratton, 1948.

<sup>6</sup> Bergenhem, B., and Fahraeus, R., *Z. Ges. Exp. Med.*, 1936, 97, 555.

the blood collected in separate oxalated tubes. The fetal and maternal livers, spleens, kidneys, hearts and lungs were perfused *in situ* to wash out as much blood as possible and then removed and frozen on dry ice. When all the tissues were removed and frozen they were weighed, then ground in a porcelain mortar and 0.9% NaCl was added to obtain suspensions varying from 5 to 30%. These suspensions were centrifuged and the supernatant fluid was used in subsequent tests.

In the experiments reported here the activity of each extract was tested on both fetal and maternal red cells. In several runs a suspension of red cells was used from a species other than the one from which the tissue extracts were prepared. In all instances, the cells were washed three times with 10 to 12 volumes of physiological saline and used in the test as a 1% suspension.

The supernatant fluid of the tissue suspension was diluted by pipetting into several 12 x 75 mm test tubes 1.0, 0.5, 0.25 and 0.10 ml of the extract and making them up to 1.0 ml with 0.9% saline. Several tubes containing 1.0 ml saline were prepared to serve as controls. One ml of the red cell suspension was then added to these tubes. Since some fetal liver extracts, despite *in situ* perfusion, possessed interfering color it was necessary to set up a series of tubes containing various dilutions of the extract together with 1.0 ml of saline instead of the red cell suspensions. These tubes served as color controls. The tubes were placed in an incubator (38°C) and at one-half hour intervals they were shaken. After 2½ to 4 hours incubation they were removed and centrifuged at 2,000 RPM for 5 minutes. The degree of hemolysis was determined visually by the amount of color in the supernatant liquid (corrected for by the "color controls") and by the amount of sediment. The usual grades, 4, indicating complete hemolysis, to 0, for none, were employed.

**Results.** As illustrated in Table I, extracts of fetal guinea pig liver (crown-rump size from 6 to 9 cm) lysed *in vitro*, and within 4 hours, suspensions of red cells from the

blood of the same fetus, as well as from that of the mother. They also exhibited this lytic action on red cells from the male of the same species, as well as against a red cell suspension of rat blood. This activity could be demonstrated in dilutions equivalent to 0.5% of the original suspensions. Extracts of maternal guinea pig liver exhibited detectable activity only in relatively high concentrations and, in the experiments reported here, failed to lyse within the 4 hour period the red cells of the rat. Extracts of other fetal tissues (Table II), with the possible exception of the spleen, did not show as high activity as that found for liver, nor were the fetal-maternal differences as consistent as those found for liver. Extracts of fetal guinea pig spleen, in 2 experiments, and fetal kidney, in one experiment, possessed greater lytic activity than the corresponding maternal extracts. In the rat, fetal liver and spleen possessed greater activity than the maternal organs. Extracts of adult rat lung and adult guinea pig lung appeared to produce more lysis than the corresponding fetal organs. However, in these experiments very little lysis was produced after 4 hours.

Boiling the extract of fetal guinea pig liver for one minute completely destroyed the activity (Table I.). The precipitate of such a heated extract caused a small amount of hemolysis at the end of 4 hours, indicating that a heat-stable substance was carried down by the precipitate. Heating for one hour at 56°C destroyed about 75% of the activity. The addition of fresh serum to such a heat-inactivated extract did not restore the activity. Apparently complement, in the classical sense, is not involved in the hemolysis. Allowing the extracts to stand for one hour with heated (to 56°C) or fresh serum before the red cells are added reduces the amount of hemolysis. This may mean that an anti-hemolytic factor that is not a protein is present in the serum.

If the complete system (extracts plus red cell suspension) is allowed to stand overnight, a marked increase in hemolysis is observed and, in some experiments with liver extracts, the fetal-maternal differences are obscured.

Although heat-labile factors are responsible for the greater portion of the activity reported in these experiments a small amount is found to be due to heat-resistant substances. This brings up the question of their probable nature.

(3) In addition to the two hemolytic systems briefly described above there is evidence for the existence of a third system. Freeman and Johnson<sup>13</sup> and Loewy, *et al.*<sup>14</sup> have shown that an increase in soaps in chyle, particularly during fat absorption after a meal high in fats, can be large enough to bring about a degree of hemolysis on entering the blood. The fetal guinea pig liver, at the stages studied, has a very high fat content<sup>15</sup> and it is conceivable that as a result there is a sufficient increase in the soap content to account for the heat-stable hemolytic agent.

The fact that serum heated, as well as fresh, inhibits the activity of the lytic agents points to the probability that this may be part of an *in vivo* counteracting mechanism.

Bearing on the general phenomena reported here is the recent observation of Gross<sup>16,17</sup>

to the effect that saline extracts from spontaneous mouse mammary carcinomas and human cancer frequently hemolyse red cells *in vitro*. With the former preparation hemolysis was observed after 2 to 3 hours incubation whereas with the latter it became evident only after 24 hours. Weil<sup>10</sup> also reported a similar study in which he observed that non-necrotic tumors behaved like normal tissue while necrotic tissue apparently produced a different kind of a lytic agent. Both Weil and Gross offer their findings as the basis for an explanation of the anemias which frequently occur during malignancies.

It should be noted that in respect to time of incubation needed to produce lysis fetal liver extracts resemble extracts of spontaneous mouse mammary carcinoma.

**Summary.** (1) A saline extract of the liver of fetal guinea pig possesses greater hemolytic activity than a similarly prepared extract of maternal liver, as determined after 4 hours incubation at 38°C.

(2) The activity, for the most part, is due to a heat-sensitive agent and, to a lesser extent, to a heat-resistant substance.

(3) The lytic action of such extracts is not species-specific. It will lyse red cells from the same fetus, as well as those from the mother or from another species.

<sup>13</sup> Freeman, L. W., and Johnson, V., *Am. J. Physiol.*, 1940, **130**, 723.

<sup>14</sup> Loewy, A., Freeman, L. W., Marchello, A., and Johnson, V., *Am. J. Physiol.*, 1943, **138**, 320.

<sup>15</sup> Imrie, C. G., and Graham, S. G., *J. Biol. Chem.*, 1920, **44**, 243.

<sup>16</sup> Gross, L., *Proc. Soc. Exp. Biol. and Med.*, 1947, **65**, 202.

<sup>17</sup> Gross, L., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 656.

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### Amino Acid Composition of Morphological Fractions of Rat Livers and Induced Liver Tumors.\* (17480)

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In previous studies,<sup>1,2</sup> comparisons were made of the chemical compositions of the nuclear, large granule (mitochondria), small granule (microsome), and supernatant fluid fractions of the livers from normal rats, the livers of rats fed the hepatic carcinogen 4-

dimethylaminoazobenzene, and the liver tumors induced by this dye. Ingestion of the dye resulted in altered levels of protein, nucleic acids, and riboflavin in certain fractions. In particular, increased levels of protein in the nuclear fraction and decreased

TABLE II.  
Hemolytic Action of Saline Extracts of Other Tissues Using Maternal Red Cell Suspensions.

		Dilution (see text)			
		15.0	7.5	3.8	1.5
A.	Guinea pig				
1.	Spleen				
	Fetal	++	+	0	0
	Maternal	+	+	0	0
2.	Kidney				
	Fetal	+	±	0	0
	Maternal	±	0	0	0
3.	Lung				
	Fetal	0	0	0	0
	Maternal	++	++	+	+
B.	Rat				
1.	Liver				
	Fetal	++++	+	±	0
	Maternal	++	0	0	0
2.	Spleen				
	Fetal	++++	++++	+++	+
	Maternal	±	±	0	0
3.	Lung				
	(Homogenized in blender)				
	Fetal	++	++	+	±
	Maternal	+++	+++	++	+

system is heat-sensitive and is inactivated at 56°C. (2) That lysins can be obtained from tissues other than the spleen has been shown by Metchnikoff,<sup>9</sup> Weil,<sup>10</sup> Maegraith, Findlay and Martin,<sup>11</sup> and Ponder.<sup>12</sup> Weil found that kidney and liver extracts contained lysins and that their activity was inhibited by serum. He also pointed out that the lytic activity of some extracts may be derived from residual blood in the tissue. In the experiments reported here it was extremely difficult to obtain blood-free saline extracts of fetal liver. However, it should be pointed out that the lytic action of fetal liver was equally as potent against the red cells of the same fetus as against the maternal or rat blood cells. Maegraith, Findlay and Martin<sup>11</sup> have recently described

a heat-sensitive agent found in a variety of human and animal tissues. This agent produces lysis after 24 hours of incubation at 37°C. Their lysin is inactivated at 80°C in 5 minutes and they report it to be species-specific. Ponder<sup>12</sup> has been able to confirm in part the observations of Maegraith, *et al.*, but he finds that extracts he prepared are not species-specific. Weil<sup>10</sup> did not determine whether his extracts lysed the red cells of other species. The heat-sensitivity and lack of species-specificity suggest that the predominant factor in the fetal liver is similar, as far as those two characteristics are concerned, to the agent found by Ponder,<sup>12</sup> but differ as to the time required to produce lysis. This may be due to a higher concentration of the factor in fetal liver. The results also suggest that it may be lysolecithin, or some closely related substance. The inhibitory action of iodoacetate in preventing the accumulation of such lytic agents might be accounted for by its activity on the enzyme responsible for the production of the lysolecithin.

<sup>7</sup> Singer, K., *Am. J. Med. Sc.*, 1940, **199**, 466.

<sup>8</sup> Singer, K., *J. Clin. Inv.*, 1941, **20**, 153.

<sup>9</sup> Metchnikoff, cited by Weil.<sup>10</sup>

<sup>10</sup> Weil, R., *J. Med. Research*, 1907, **16**, 287.

<sup>11</sup> Maegraith, B. G., Martin, N. H., and Findlay, G. M., *Brit. J. Exp. Path.*, 1943, **24**, 58.

<sup>12</sup> Ponder, E., *J. Gen. Physiol.*, 1944, **27**, 483.

TABLE I.  
Distribution of Protein and Nucleic Acids in the Tissue Fractions.

Livers from rats fed			
Fraction	Basal diet	Basal diet + dye	Rat liver tumors
Mg protein per g fresh tissue (to the nearest whole number)			
Whole homogenate	123	106	119
Nuclei	16	16	42
Large granules	41	29	14
Small "	18	16	12
Supernatant fluid	47	41	49
Recovery	122	102	117
Mg of desoxypentosenucleic acid per g of fresh tissue			
Whole homogenate	1.94	2.10	5.57
Nuclei	1.89	2.01	5.00
Large granules	0.00	0.00	0.49
Small "	0.00	0.00	0.10
Supernatant fluid	0.00	0.00	0.24
Recovery	1.89	2.01	5.83
Mg of pentosenucleic acid per g of fresh tissue			
Whole homogenate	5.70	3.57	6.89
Nuclei	0.49	0.25	1.76
Large granules	1.80	1.06	0.76
Small "	1.78	0.97	1.33
Supernatant fluid	1.11	0.96	2.47
Recovery	5.18	3.24	6.32

*vacuo* first over  $\text{CaCl}_2$  and finally over  $\text{H}_2\text{SO}_4$ . This procedure was carried out quantitatively and the yields were determined by direct weighing. Analyses for ash, lipid, and glycogen indicate that approximately 97% of such preparations is a mixture of protein and nucleic acids.<sup>9</sup> Nitrogen was determined by the Dumas method.<sup>§</sup>

For the microbiological amino acid determinations, 40 mg of the dried preparations were autoclaved in 3 ml of 3 *N* HCl at 121°C for 16 hours, neutralized, diluted to 100 ml and appropriate aliquots were used for each assay. Previous studies have shown that the amino acids determined are essentially stable to this treatment. Leucine, valine, phenylalanine, and glutamic acid were determined with *Lactobacillus arabinosus* 17-5 as the test organism; arginine, threonine, and histidine with *Streptococcus faecalis* R as the test organism; lysine, methionine, aspartic acid, proline, serine, isoleucine, tyrosine, and cystine with *Leuconostoc mesenteroides* P-60; and glycine with *Leuconostoc citrovorum* 8081 as the test organism. All of the assays

were carried out with a final volume of 2 ml per tube and with the assay conditions and media summarized in a recent publication.<sup>10</sup> Liver extract was also included in the medium for *Leuconostoc citrovorum* 8081.<sup>11</sup> Tryptophan was determined on separate 10-30 mg samples of the protein preparations by the Bates method as described by Graham *et al.*<sup>12</sup> Calculated on a fresh weight basis similar to that indicated in Table I for protein and nucleic acids, the sum of the amounts of each amino acid in the 4 fractions was in good agreement with the amount found in the unfractionated homogenate.

**Results and Discussion.** In the preliminary study, it was found that, when rats were killed by decapitation without ether anesthesia, the perfusion removed about 0.05 ml of blood per gram of liver. If the rats were killed

<sup>9</sup> Miller, E. C., and Miller, J. A., *Cancer Research*, 1947, **7**, 468.

<sup>10</sup> Schweigert, B. S., Guthneck, B. T., Kraybill, H. R., and Greenwood, D. A., *J. Biol. Chem.*, 1949, **180**, 1077.

<sup>11</sup> Sauberlich, H. E., and Baumann, C. A., *J. Biol. Chem.*, 1948, **176**, 165.

<sup>12</sup> Graham, C. E., Smith, E. P., Hier, S. W., and Klein, D., *J. Biol. Chem.*, 1947, **168**, 711.

<sup>§</sup> Clark Microanalytical Laboratories, 104½ West Main Street, Urbana, Ill.

quantities in the large and small granule fractions were observed.<sup>1</sup> Similar changes, but more extreme, were found in the proteins of the tumor fractions.<sup>2</sup> With the aid of microbiological methods developed for the determination of amino acids,<sup>3,4</sup> we have now determined the levels of 17 amino acids in the total proteins of the intracellular fractions from the livers of rats fed diets with or without 4-dimethylaminoazobenzene and from liver tumors. Isolation of the morphological parts of the cell permitted the detection of alterations in the composition of the cell which would have been obscured if only the whole cell proteins had been analyzed.

**Experimental.** Although the livers fractionated for the amino acid determinations were first perfused with saline, the degree to which contamination of the liver proteins by blood proteins might otherwise occur was determined in a preliminary study. This was calculated for each of several rats from counts of the red blood cells in the heart blood and in the perfusion fluid from the liver. The rats were killed by ether or by decapitation, and, in each case, the inferior vena cava was cannulated cephalic to the liver and ligated just caudal to the liver. The portal vein was then severed and the liver carefully excised. Isotonic NaCl was introduced through the cannula and allowed to flow out through the portal vein into a collecting vessel. The livers were weighed before and after perfusion. The livers that were fractionated were perfused in a similar manner *in situ*. For the fractionations, two groups of 4 male rats† weighing 180 to 200 g were fed *ad*

*libitum*, a basal diet,<sup>5</sup> containing 12% of casein and 1.2 mg of riboflavin per kg. The diet for one group also contained 0.06% of 4-dimethylaminoazobenzene. After 4 weeks, the rats were killed with ether and their livers were immediately perfused. The tumor tissue was collected from the livers of 20 rats which had been fed the diet containing 0.06% of 4-dimethylaminoazobenzene for 4 to 5 months. Although only liver tumors which were less than 1 cm in diameter and which showed no gross signs of degeneration were used, histological examination showed that the tumors contained small areas of necrosis and connective tissue and numerous leucocytes.<sup>2</sup> The tumor-bearing livers were not perfused. These tumors were nearly white and their color was unchanged upon perfusion of the liver. This would now be expected from the recent demonstration that liver tumors are supplied only with arterial blood.<sup>6</sup> Furthermore, omission of this step considerably decreased the time required for the collection of the tumors. The livers or tumors were then pooled, forced through a plastic tissue mincer, homogenized in 0.88 M sucrose solution, and fractionated by differential centrifugation as described previously.<sup>1,2,7</sup> The perfusion, centrifugation, and all other operations prior to the precipitation of the proteins were performed at 3°C. Each fraction was suspended in sucrose solution, and aliquots from each fraction and the unfractionated homogenate were analyzed for nucleic acids.<sup>8</sup> The proteins in the remainder of each fraction and from an aliquot of the original homogenate were precipitated with trichloroacetic acid at a final concentration of 9% and washed once with 1 M acetate buffer (pH 5.0) and twice with 95% ethanol. Following extraction for 48 hours with ethanol at 60°C in a Soxhlet apparatus, the proteins were dried in weighing bottles *in*

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† Predoctorate Research Fellow, National Cancer Institute, 1948-49.

<sup>1</sup> Price, J. M., Miller, E. C., and Miller, J. A., *J. Biol. Chem.*, 1948, **173**, 345.

<sup>2</sup> Price, J. M., Miller, J. A., Miller, E. C., and Weber, G. M., *Cancer Research*, 1949, **9**, 96.

<sup>3</sup> Snell, E. E., *Adv. in Prot. Chem.*, 1945, **2**, 85.

<sup>4</sup> Schweigert, B. S., and Snell, E. E., *Nutr. Abst. and Rev.*, 1946-47, **16**, 497.

‡ Holtzman Rat Co., Madison, Wis.

<sup>5</sup> Miller, E. C., Miller, J. A., Kline, B. E., and Rusch, H. P., *J. Exp. Med.*, 1948, **88**, 89.

<sup>6</sup> Breedis, C., and Young, G., *Fed. Proc.*, 1949, **8**, 351.

<sup>7</sup> Price, J. M., Miller, E. C., Miller, J. A., and Weber, G. M., *Cancer Research*, 1949, **9**, 398.

<sup>8</sup> Schneider, W. C., *J. Biol. Chem.*, 1945, **161**, 293.

TABLE III.  
Amino Acid Composition of the Proteins of the Tissue Fractions  
(Expressed as % of the nucleic acid-free proteins)

Fraction	Proline	Glycine	Aspartic acid	Glutamic acid	Cystine	Tyrosine	Serine
Livers from rats fed basal diet 4 wk							
Whole homogenate	4.00	4.01	8.38	9.87	.58	3.99	4.05
Nuclei	4.37	5.62	8.06	8.85	.46	3.71	3.89
Large granules	3.90	4.16	8.17	9.20	.52	3.87	4.08
Small "	3.78	3.37	7.79	9.20	.55	4.09	3.98
Supernatant fluid	4.20	3.71	7.99	10.28	.71	3.70	3.76
Livers from rats fed basal diet $\frac{1}{2}$ dye 4 wk							
Whole homogenate	4.21	3.97	7.61	9.81	.64	3.93	3.95
Nuclei	4.24	5.83	6.87	11.35	.46	3.45	4.19
Large granules	4.05	3.90	7.13	10.44	.49	3.86	3.94
Small "	3.64	3.45	7.54	9.20	.59	4.19	4.08
Supernatant fluid	3.81	3.74	8.20	11.10	.85	3.54	3.87
Liver tumors induced by feeding dye 4 to 5 mo.							
Whole homogenate	4.07	4.86	7.72	11.11	.74	3.66	4.30
Nuclei	4.24	6.93	7.26	10.83	.60	3.63	4.28
Large granules	3.61	4.10	7.79	9.68	.75	4.02	4.29
Small "	3.41	3.56	7.80	9.79	.63	3.68	4.34
Supernatant fluid	3.66	3.58	8.35	11.41	.59	3.99	5.00

samples contained 14.5 and 14.9% of nitrogen, respectively. Through an oversight, none of the protein sample from the whole homogenate of the livers of the rats fed the dye was reserved for nitrogen analysis. The 17 amino acids determined accounted for 85 and 79% of the nitrogen in the nucleic acid-free proteins from the whole homogenates of the livers of the rats fed the basal diet and of the liver tumors. Analyses for alanine with *Leuconostoc citrovorum* indicated that the liver proteins from the rats fed the basal or dye-containing diets and the tumors contained approximately 6% of alanine. The inclusion of alanine increased the nitrogen recoveries to 91 and 86%, respectively, for the nucleic acid-free proteins from the livers of the rats fed the basal diet and the liver tumors.

Table IV lists those amino acids whose concentrations in the nucleic acid-free proteins from the various fractions differed from their concentrations in the proteins from the unfractionated tissues by 15% or more. In general, the proteins from the nuclear fractions contained less histidine, tryptophan, cystine, and phenylalanine and about 40% more glycine than the proteins from the unfractionated tissues. The proteins from the large

granules usually had a relatively low concentration of histidine, while those from the small granules generally had a lower concentration of methionine, proline, and glycine and a higher concentration of tryptophan than the whole homogenate proteins.

In each case the supernatant fluid proteins contained more cystine than whole liver protein. Melampy<sup>15</sup> has shown that the whole nuclei of chicken erythrocytes contain less histidine, tryptophan, and phenylalanine than the cytoplasm; no analyses for cystine or glycine were reported. The livers of the rats which had been fed the dye for 4 weeks contained 20 to 33% higher concentrations of glutamic acid in the nuclear proteins, arginine in the large granule proteins, and cystine and tryptophan in the supernatant fluid proteins than were found in the corresponding fractions from the normal livers, Table V. The tumor proteins differed from the normal liver proteins even more than did those from the livers of the rats fed the dye. The methionine contents of the proteins from each fraction were decreased by 25 to 41% while the cystine contents were increased by 15 to 44%. However, the levels of cystine in these proteins

<sup>15</sup> Melampy, R. M., *J. Biol. Chem.*, 1948, **175**, 589.



with ether and not decapitated, approximately 0.2 ml of blood was removed per gram of liver. The livers from the rats killed with ether had the same weight before and after perfusion while the livers from decapitated rats gained approximately 15% in weight; thus in either case the saline remaining in the portal vessels accounted for about 20% of the fresh weight of the livers. Therefore, the total proteins from the unperfused livers of rats killed by decapitation or by ether anesthesia would contain approximately 6 or 22%, respectively, of blood proteins, even assuming that the blood contained as little as 20% of protein and the perfused liver as much as 15% of protein. These results are in agreement with those of Grund<sup>13</sup> on the perfusion of dog liver. The levels of total protein, desoxypentosenucleic acid, and pentosenucleic acid in the whole homogenates and in each of the fractions (Table I) are in agreement with previous results.<sup>1,2</sup> Subsequent studies have shown that increasing the casein content of the basal diet from 12 to 24% does not alter the level or distribution of protein in the livers of adult rats.<sup>14</sup> In order to express the amino acid data on a protein basis, the "nucleic acid-free protein" has been calculated by subtracting the quantities of the two nucleic acids from the amount of protein in the same fraction.

The levels of arginine, histidine, lysine, leucine, valine, isoleucine, threonine, tryptophan, phenylalanine, and methionine in the nucleic acid-free proteins from the whole homogenates and from each of the fractions are presented in Table II. Similar data for proline, glycine, aspartic acid, glutamic acid, cystine, tyrosine, and serine are shown in Table III. By Dumas analysis, the protein-nucleoprotein preparation of the whole homogenate from the livers of rats fed the basal diet contained 14.5% nitrogen and the whole homogenate from the liver tumors contained 15.1% nitrogen. By calculation, the nucleic acid-free proteins from these

TABLE II. Amino Acid Composition of the Proteins of the Tissue Fractions. (Expressed as % of the nucleic acid-free proteins).

Fraction	Arginine	Histidine	Lysine	Leucine	Valine	Isoleucine	Threonine	Tryptophan	Phenylalanine	Methionine
Whole homogenate	6.44	3.20	8.84	10.66	5.76	4.92	4.26	1.49	4.97	2.91
Nuclei	7.15	2.68	9.56	10.10	5.22	4.62	4.17	1.22	3.92	2.60
Large granules	5.57	2.69	8.35	10.45	5.50	5.50	3.97	1.54	4.82	2.98
Small "	6.22	2.97	8.20	9.80	5.00	4.36	4.09	1.85	4.70	2.48
Supernatant fluid	5.59	2.92	8.12	10.10	5.15	4.66	3.76	1.05	4.42	2.66
Livers from rats fed basal diet + dye for 4 wk										
Whole homogenate	6.10	2.75	8.66	10.40	5.14	4.63	4.07	1.52	4.51	2.74
Nuclei	7.57	2.51	8.68	9.62	4.93	4.38	3.90	1.36	3.79	2.55
Large granules	6.71	2.55	7.94	10.65	5.64	4.77	4.15	1.42	4.48	2.71
Small "	6.14	2.79	7.86	9.90	5.13	4.40	3.79	2.15	4.50	2.07
Supernatant fluid	6.12	2.97	7.89	10.73	5.35	4.85	3.99	1.40	4.36	2.98
Liver tumors induced by feeding dye 4 to 5 mo.										
Whole homogenate	6.32	2.55	8.45	9.45	4.80	4.53	3.92	1.39	3.74	1.95
Nuclei	6.98	2.26	8.06	8.52	4.59	3.91	3.64	1.09	3.30	1.54
Large granules	5.90	2.20	7.94	9.80	5.15	4.53	3.27	1.68	3.90	2.14
Small "	5.38	2.40	7.53	9.15	4.80	4.25	3.64	1.62	3.87	1.89
Supernatant fluid	5.83	2.71	8.15	9.58	5.25	4.30	4.30	1.03	3.89	2.00

<sup>13</sup> Grund, G., *Z. Biol.*, 1910, **54**, 173.

<sup>14</sup> Price, J. M., Miller, E. C., Miller, J. A., and Weber, G. M., *Cancer Research*, in press.

TABLE V.  
Summary of Amino Acids Which Occurred in Livers of Rats Fed Dye and in Tumors at Levels That Differed from Normal Liver by 15% or More.  
(Expressed as % higher (+) or lower (—) in concentration than in normal liver).

Fraction	Livers from rats fed basal diet + dye	Rat liver tumors			
Whole homogenate		Methionine	—33		
		Cystine	+28		
		Glycine	+21		
		Phenylalanine	—25		
		Valine	—17		
Nuclei	Aspartic acid —15	Methionine	—41	Histidine	—16
		Cystine	+30	Isoleucine	—15
		Glycine	+23	Lysine	—16
		Phenylalanine	—16	Leucine	—16
		Glutamic acid	+22		
	Glutamic acid +28				
Large granules	Arginine +20	Methionine	—28	Histidine	—18
		Cystine	+44	Isoleucine	—18
		Phenylalanine	—19	Threonine	—18
Small granules	Tryptophan +16				
	Methionine —17	Methionine	—24	Histidine	—19
		Cystine	+15		
		Phenylalanine	—18		
Supernatant fluid	Tryptophan +33				
	Cystine +20	Methionine	—25		
		Cystine	+25		
		Serine	+33		

fractions of normal liver. In addition, the concentrations of glutamic acid and glycine in the nuclear proteins and of serine in the super-

natant fluid proteins were higher in the tumor tissue than in normal liver.

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### Utilization of Guanine by the C57 Black Mouse Bearing Adenocarcinoma Eo771.\* (17481)

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The effectiveness of the triazole analogue of guanine, azaguanine, in inhibiting the growth of subcutaneous transplants of mammary adenocarcinoma Eo771<sup>1</sup> in mice of the C57 black strain has been reported by Kidder,

Dewey, Parks and Woodside<sup>2</sup> and confirmed for this tumor.<sup>3</sup> It was suggested by Kidder and co-workers<sup>2</sup> that their results indicated that the tumor tissue utilizes guanine for nucleic acid synthesis while normal tissue does not.

\* The authors wish to acknowledge the support of the National Cancer Institute, United States Public Health Service, the Office of Naval Research, and the James Foundation of New York, Inc.

<sup>1</sup> Snell, G. D., Cloudman, A. M., and Woodworth, E., *Cancer Res.*, 1948, 8, 429.

<sup>2</sup> Kidder, G. W., Dewey, V. C., Parks, R. E., Jr., and Woodside, G. L., *Science*, 1949, 109, 511.

TABLE IV.  
Summary of Differences Between Amino Acid Composition of Proteins from Fractions and the Whole Homogenates Which Exceeded 15%.  
(Expressed as % higher (+) or lower (—) in concentration in the fraction).

Livers from rats fed						
Fraction	Basal diet		Basal diet + dye		Rat liver tumors	
Nuclei	Histidine	—16	Arginine	+24	Histidine	—18
	Tryptophan	—18			Tryptophan	—22
	Phenylalanine	—21	Phenylalanine	—16		
	Glycine	+40	Glycine	+47	Methionine	—21
			Glutamic acid	+17	Glycine	+43
	Cystine	—21	Cystine	—28	Cystine	—19
Large granules	Histidine	—16			Histidine	—20
					Threonine	—17
			Cystine	—23	Tryptophan	+21
				Glycine	—16	
Small granules					Arginine	—15
	Tryptophan	+24	Tryptophan	+41	Tryptophan	+17
	Methionine	—15	Methionine	—24		
			Proline	—16	Proline	—16
	Glycine	—16			Glycine	—27
				Cystine	—15	
Supernatant fluid	Tryptophan	—30			Tryptophan	—26
			Methionine	—17		
					Glycine	—26
	Cystine	+22	Cystine	+33	Cystine	+20
					Serine	+16

were relatively low and the cystine assay is less reliable than the other microbiological assays used. The concentrations of glycine and glutamic acid in the nuclear proteins and of serine in the supernatant fluid proteins were also increased by 23 to 33%, respectively. Whether the alterations found following the ingestion of the carcinogen were due to changes in the relative amounts of the various proteins or to alterations in the composition of specific proteins will require much further study.

**Summary.** 1. The perfused livers from adult rats fed either a basal diet containing 12% of casein or the same diet plus 4-dimethylaminoazobenzene for 4 weeks, and the liver tumors induced by this dye were fractionated into nuclear, large granule (mitochondria), small granule (microsome), and supernatant fluid fractions. The amounts of 17 amino acids were determined in the total proteins from the original homogenate and in each of

the fractions. The importance of using perfused livers for these analyses was demonstrated experimentally.

2. When the basal diet was fed, the proteins from the nuclear fraction contained lower concentrations of histidine, tryptophan, phenylalanine, and cystine and a higher concentration of glycine than the whole liver proteins. The large granule proteins contained less histidine, the small granule proteins less methionine and glycine and more tryptophan, and the supernatant fluid proteins less tryptophan and more cystine than the proteins of the original homogenate.

3. The livers of rats fed the dye contained higher concentrations of glutamic acid in the nuclear proteins, arginine in the large granule proteins, and cystine and tryptophan in the supernatant fluid proteins than normal livers.

4. The proteins from each fraction of liver tumor contained less methionine and more cystine than the proteins in the corresponding

in any other tissue examined, thus lending further support to our hypothesis.

Metallic Re powder was irradiated in the Oak Ridge nuclear reactor. It was dissolved in 2 ml of concentrated nitric acid and appropriately diluted for parenteral administration. This perhenic acid solution contained 2 radioactive Re isotopes,  $\text{Re}^{186}$  and  $\text{Re}^{188}$  having half lives of 90 and 18 hours, respectively, as well as a large amount of stable Re. In nearly all the experiments to be described, the 18 hour half life isotope was allowed to decay to such an extent that by the time of our experiments it was an unimportant factor in our measurements. Of necessity, however, a relatively large amount of carrier Re was given with each dose—from 3 to more than 100  $\mu\text{g}$  depending on the age of the irradiated Re. The amount of stable Re given in each case was therefore greater than a tracer quantity.

Adult albino rats weighing 250 to 400 g were fed on a diet consisting of mixed cereals, dried milk, meat scrap, yeast, and salts moistened with fresh milk. Fresh vegetables were given separately. Since we were studying only the filtration process of the thyroid, it was desirable to inhibit completely the reactions whereby thyroxine and diiodotyrosine are synthesized. To this end, thiouracil<sup>‡</sup> was added to the mixture to make a 1% concentration. For comparison with normals, however, thiouracil was omitted from the food of 8 rats.

One ml of a  $\text{HRe}^*\text{O}_4$  solution having an activity of 200,000 to 300,000 counts per minute<sup>§</sup> was injected intraperitoneally into each rat. The animals were sacrificed with  $\text{CHCl}_3$  usually 4 hours after the injection. In a number of instances in which the effect of time on the  $\text{Re}^*$  concentration of tissues was studied, rats were killed 1 to 24 hours after injection. Autopsies were performed at once and tissues prepared for analysis.

<sup>‡</sup> We thank the American Cyanamid Company for supplying the thiouracil.

<sup>§</sup> The activity of the Re shipments varied from 25,000 to 100,000 cpm per  $\mu\text{g}$  at the time of shipment as determined from data submitted with the irradiated preparations and from the catalogue of isotopes of the Atomic Energy Commission.

As much blood as possible was drained from the tissue before hashing. One to 2 g samples of blood, lung, liver and muscle were weighed into 15 mm diameter metal planchets, moistened with 10-20 drops of 1% NaOH, dried for an hour at 105°C and then at 120-130°C. The thyroid of each rat was weighed in a planchet, moistened with 1-2 drops of NaOH and dried in the same way. The samples were then ashed at 450-500°C for 18 hours. The ash was coated with 0.5-1.0 ml of 1% celloidin and the activity measured with a thin mica window Geiger counter. Appropriately diluted samples of the injected solution measured with the same apparatus were used as standards to establish the decay curve for the  $\text{Re}^*$  preparation in each series of experiments. Known amounts of  $\text{Re}^*$  were added to blood and to muscle samples and ashed as described. The maximum loss in these controls due to self absorption and possibly to some loss during ashing was found to be not more than 15% and usually less than 10%. No corrections are made for these errors in the data given.

Fig. 1 shows the distribution of  $\text{Re}^*$  in thyroid, lung, liver, muscle, and blood of 8 normal rats measured as described. Fig. 2 shows similar observations on 8 representative rats which had been under the influence of thiouracil for from 5 to 20 days before  $\text{Re}^*$  was injected. The thyroids weighed 45 to over 100 mg depending on the duration of thiouracil feeding. They were very hyperemic and hyperplastic. The thyroids of the control rats weighed 14 to 20 mg, a few being slightly enlarged and slightly hyperemic.

Four hours after  $\text{Re}^*$  injection, an average of 4.5% of the administered dose per g of tissue was found in the thyroids of normal rats, the range was 1.4 to 7.2% per g. For thiouracil treated rats which had been given 1 ml of a  $\text{Re}^*$  solution containing 3 to 12  $\mu\text{g}$  of carrier, the average concentration was 12.8% per g of thyroid with a range of 5.1 to 25.5% per g. When larger amounts of carrier were used, smaller proportions of the injected dose were found in the thyroids. For example, with 4 thiouracil treated rats a  $\text{Re}^*$  solution containing 250  $\mu\text{g}$  Re carrier

We have investigated the incorporation of isotopically labeled guanine (containing  $N^{15}$  in the 1 and 3 nitrogens and in the 2-amino group<sup>3</sup>) into the nucleic acids of this strain of mouse bearing this tumor. The guanine was administered in five intraperitoneal injections, of 2.4 mg each, over a 6 day period, beginning on the 7th day after implantation of the tumor. Inasmuch as isotopic nitrogen, particularly from the 2-amino group, can be contributed to body pool ammonia<sup>4</sup> it is not unlikely that the isotope found in the protein residue, pyrimidines and even the adenine (Table I) arose from this source. There was, however, a small but definite incorporation into the guanine of the nucleic acids, but there is no evidence of a specific uptake by the tumor since the incorporation into the guanine of the tumor tissue and into that of the non-tumorous viscera of the same mice was significant in each case.

When guanine was administered to an unspecified strain of the white rat<sup>5</sup> or to Sherman strain rats (Rockland Farms), either orally<sup>4</sup>

<sup>3</sup> Sugiura, K., Hitchings, G. H., Cavalieri, L. F., and Stock, C. C., in press.

<sup>4</sup> Brown, G. B., Roll, P. M., Plentl, A. A., and Cavalieri, L. F., *J. Biol. Chem.*, 1948, **172**, 469.

<sup>5</sup> Plentl, A. A., and Schoenheimer, R., *J. Biol. Chem.*, 1944, **153**, 203.

<sup>6</sup> Brandt, A., Roll, P. M., and Brown, G. B., unpublished data.

Incorporation of Guanine into Nucleic Acid of C57 Mice Implanted with Adenocarcinoma Eo771.

	Atom % excess $N^{15}$
Injected guanine	6.40
Viscera:	
Nucleic acids	.030
Total purines	.049
Guanine	.072
Pyrimidines	.024
Tissue residue (after extraction of nucleic acids)	.015
Tumor:	
DNA	
Total purines*	.046
Adenine†	.038
Guanine	.050
PNA	
Total purines*	.058
Adenine†	.037
Guanine	.105

\* Isolated as the copper salts after separation of the pentose (PNA) and desoxypentose (DNA) nucleic acids by the Schmidt-Thannhauser procedure.

† Calculated from  $N^{15}$  values of adenine picrate.

or intraperitoneally,<sup>6</sup> there was no significant incorporation of the guanine into the tissue nucleic acids. The species difference in the purine metabolism of the Sherman rat and the C57 black mouse (bearing adenocarcinoma Eo771) makes it necessary to be conscious of other such possibilities in mammalian purine metabolism.

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## Behavior of Thyroid Toward Elements of the Seventh Periodic Group. II. Rhenium.\*† (17482)

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On the basis of speculations made in a previous report<sup>1</sup> in which we suggested that

\* A report of this work was made before the American Society of Biological Chemists at Detroit, May 1948. *Fed. Proc.*, 1949, **8**, 182.

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not only iodine but all the elements of the seventh periodic group are selectively filtered by the thyroid, we have extended our work to include rhenium. As with Cl, Br, I, Mn and At, we find that Re also accumulates in the thyroid in greater concentration than

<sup>1</sup> Baumann, Emil J., and Metzger, N., *Proc. Soc. Exp. Biol. and Med.*, 1949, **70**, 536.

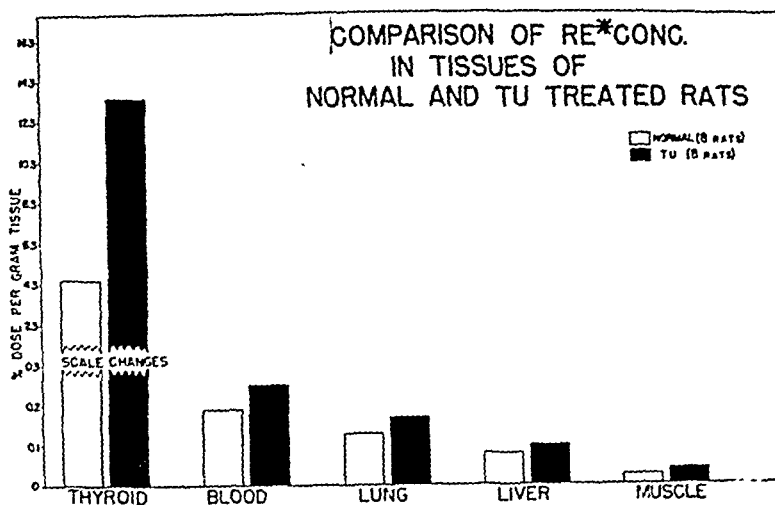


FIG. 3.

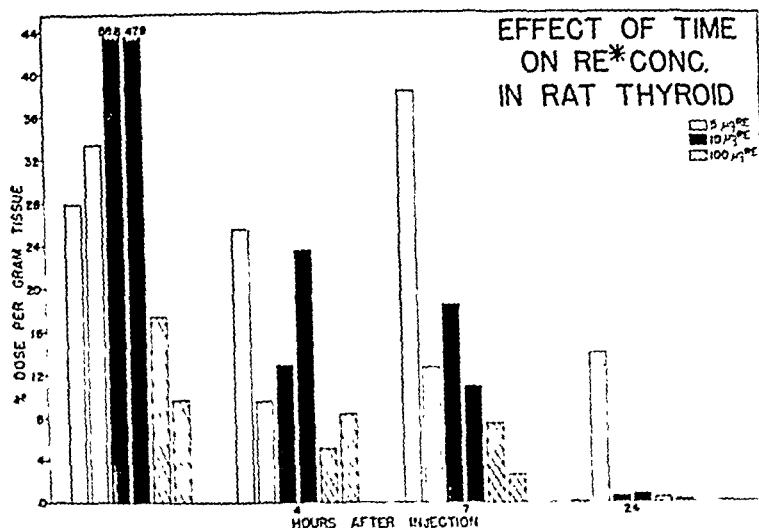


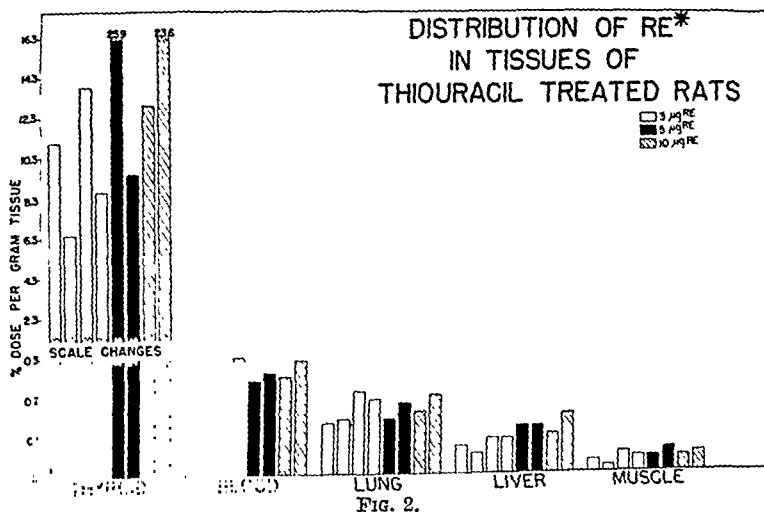
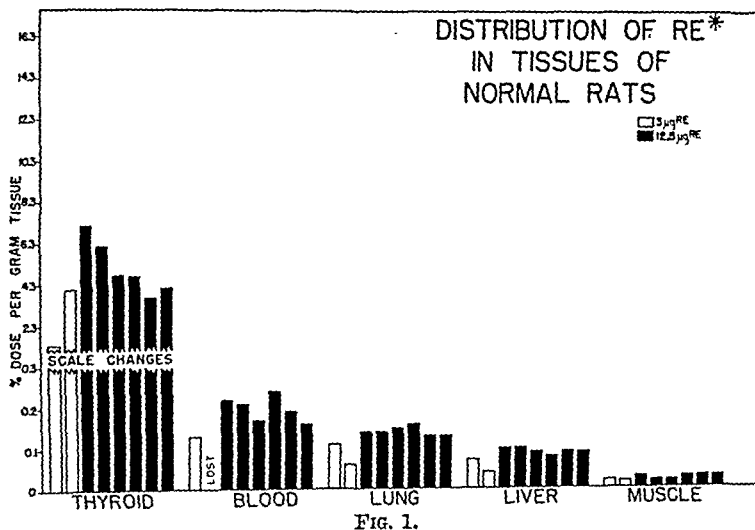
FIG. 4.

The ratio of the Re\* concentration in the thyroid to that in the blood is invariably large, 25 to 100 or more; while the ratio of Re\* concentration of other tissues to blood is always less than one. It follows, therefore, that the process whereby such a high thyroid Re\* content comes about is an active selective filtration, and not simply a passive reflection of the amount of Re\* in the blood passing through this organ.

In view of the common origin of both thyroid and lung from the primordial gill clefts, it is worthy of note that the Re\* concentra-

tion of lung is exceeded only by that of the thyroid.

To determine the effect of time on the distribution of the Re\* in the body after injection of a single dose, 3 experiments were performed. In each case 1 ml of a Re\* solution was injected intraperitoneally into 8 thiouracil fed rats. At 1, 4, 7 and 24 hours after injection 2 animals were sacrificed. The amount of carrier given per rat in these 3 experiments was 5, 10 and 100 µg and the activity of the preparations per ml was 270,000, 282,000, and 431,000 c/m, respec-



per ml was injected; 4 hours later, the thyroids were found to contain only 2.2 to 4.2% of the dose per g of tissue. Since the capacity of any tissue to take up any substance is limited, it is to be expected that with increasingly large amounts, a smaller fraction of the administered dose will be retained. As with iodine, the percentage of administered Re\* that can be taken up varies in a general way, directly with the mass of thyroid and inversely with amount of carrier.

The amount of Re\* found in other tissues was very much smaller—only 0.5% to 5% of the injected Re\* per g of tissue. The Re\*

concentrations of blood, liver, lung and muscle of the thiouracil treated rats differed little from the corresponding concentrations found in the control group. On the average, lung of normal rats contained 0.13% of the injected Re\* per g and in thiouracil fed rats 0.16%. Corresponding average figures for liver were 0.08% and 0.10%, and for muscle 0.03% and 0.04%, respectively. In all, experiments were made on 41 thiouracil fed rats with similar results in every case. Fig. 3 shows a comparison of the average Re\* uptake of the 8 normal and the 8 representative thiouracil fed rats shown in Fig. 1 and 2.

# Immunologic Response of Hamsters to Influenza Virus Strains.\* (17483)

M. M. SIGEL, E. G. ALLEN, D. J. WILLIAMS AND A. J. GIRARDI  
(Introduced by W. Henle)

From The Children's Hospital of Philadelphia, (Department of Pediatrics, and the Division of Virology, Department of Preventive Medicine and Public Health, School of Medicine, University of Pennsylvania).

Studies on strains of influenza virus isolated from the 1947 epidemic<sup>1</sup> were complicated by irregular reactions obtained in hemagglutination-inhibition tests with chicken sera (e.g., nonspecific inhibition). In view of these difficulties, it was deemed desirable to supplement the antigenic studies with complement-fixation tests. Since chicken sera are unsatisfactory for this purpose, antiserum had to be prepared in another species. The hamster was selected because of its ability to develop antibodies to influenza virus following inoculation by the intranasal route.<sup>2</sup> The results obtained with hamster sera are the subject matter of this paper.

**Methods and Materials. Viruses.** The following virus strains were used:

PR8 and Lee

L<sub>3</sub>47, L<sub>7</sub>47, and L<sub>8</sub>47

FM<sub>1</sub><sup>3</sup>

L<sub>4</sub>49 and L<sub>8</sub>49<sup>4</sup>

FJS (type A) and Warner (type B)<sup>†</sup>

**Antigens.** Allantoic fluids harvested 48 hours after intraallantoic inoculation of 10<sup>-3</sup> or 10<sup>-4</sup> dilution of seed virus which were centrifuged at 2,000 rpm for 10 minutes and dialyzed overnight against 20 parts of phosphate buffer saline were used as the "virus-bound" antigen. Obviously these preparations contained some soluble antigen. No

attempt was made to separate the two antigens from the fluids. Ten or 20% suspensions of allantoic membrane were centrifuged at 2,000 rpm for 10 minutes, and following removal of the virus (as determined by absence of hemagglutination) by either centrifugation at 13,000 rpm for one hour or absorption with red cells, or a combination of both procedures, the supernatants were used as the soluble antigen. Suspensions of uninfected membranes were used as control antigens.

**Sera.** Hamsters were inoculated by the intranasal route with 0.1-0.2 cc of active infected allantoic fluids containing virus. Fifteen days later they were bled either with needle and syringe from the heart or by incision of the thoracic cavity and heart, allowing blood to run through funnel into tube. The sera were inactivated by heating at 56°C for 30 minutes. Before being used in the hemagglutination-inhibition test, they were absorbed with human or chicken erythrocytes, depending on which red cells were to be used in the test.

**Hemagglutination-inhibition Test.** The procedure for performing the hemagglutination-inhibition test has been described previously.<sup>5</sup> The tests were read after 30-45 minutes of incubation at room temperature. In the presence of hamster sera—especially in the lower dilutions—there was a tendency on the part of the cells to settle out quickly, so that the tubes with agglutinated cells showed patterns consisting of fuzzy, serrated rings, or clumps of cells against a background of a more or less smooth film of cells (the kind of film usually observed on the bottom of tubes showing agglutination of cells in the presence of human or chicken sera).

\* This research was conducted under contract with the Office of Naval Research.

<sup>1</sup> Sigel, M. M., Shaffer, F. W., Wiener Kirber, M., Light, A. B., and Henle, W., *J. Am. Med. Assn.*, 1948, 136, 437.

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<sup>3</sup> Rasmussen, A. J., Stokes, J., and Smadel, J. E., *Am. J. Hyg.*, 1948, 47, 142.

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<sup>5</sup> Sigel, M. M., *J. Immunol.*, 1949, 62, 81.



TABLE I.  
 Excretion of Injected Re\* (%).

	Re injected		Urine			Feces		
	Carrier, mg	Re* activity, cpm, $\times 1000$	1st day	2nd day	Total	1st day	2nd day	Total
Rat 12a	0.010	309	84.4	0.7	85.1			
" 12b	0.010	309	79.4	2.2	81.6			
Rabbit 14b	0.50	1310	94.8	1.1	95.9	.1	.8	.9
" 16a	1.00	492	79.8	12.4	92.2	.0	.0	.0
" 16b	1.00	492	92.1	2.8	94.9	.0	.0	.0

tively. The uptake of Re\* by the thyroids is shown in Fig. 4.

In all tissues examined, the greatest concentration of Re\* usually appeared at the one hour interval, decreasing rather slowly to 7 hours after injection, and then more rapidly; so that 24 hours after, not more than  $\frac{1}{3}$  and usually less than  $\frac{1}{10}$  of the amount found one hour after the injection remained. After another day, the tissue activity was little more than at background levels. Although there was considerable variation among individuals (*e.g.* see Fig. 4 at the 24 hour interval) this general statement of our findings held whether 5, 10, or 100  $\mu$ g of carrier were given. Again, with the larger doses as, *eg.*, when 100  $\mu$ g of carrier Re were given, the percentage of the dose found in the thyroid and other tissues was much less than with the smaller amounts of carrier.

From these data one may assume that Re is rapidly excreted. From the urines of 2 rats, 85 and 82% of an intraperitoneal Re injection was recovered in 2 days of which all except 1-2% was excreted in the first day. Because of the difficulty of collecting quantitatively the little urine excreted by a rat, similar experiments were made with 3 rabbits. Bladders were compressed at the end of each day. Here 96, 92, and 95% of the injected Re was recovered in 2 days. The feces of 2 rabbits showed negligible radioactivity, while 0.9% of the injected dose was recovered in the feces of the 3rd rabbit. Contamination of feces by urine could not be excluded. The details of these tests are given in Table I.

Total recovery of injected Re\* was attempted on 2 rats 3.5 hours after intraperitoneal injection of 1 ml of a Re\* solution containing 13  $\mu$ g of carrier Re, having an activity of 125,000 c/m. The 2 rats were sacrificed with  $\text{CHCl}_3$  and tissues prepared for analysis. Only 72% and 85%, respectively, were recovered. The Re\* was distributed as follows:

	Rat 1, %	Rat 2, %
Thyroid	1.09	1.23
Liver	1.32	1.24
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A large part of the unrecovered rhenium can be accounted for by the loss of urine during anesthetization. In general, the distribution is similar to that known for chlorine.

**Summary.** Rhenium is preferentially and actively filtered from the blood by the thyroid. After injecting a single dose, the Re\* concentration in that organ is 25 to 100 or more times greater than that found in any other tissue. These findings therefore add support to our hypothesis that the thyroid will selectively filter from the blood all the elements of the seventh periodic group. The greatest concentration of Re\* in tissues occurs within one hour. For several hours it decreases slowly and then more rapidly so that more than 90% is recovered from the urine in 24 hours.

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*Summary.* Rhenium is preferentially and actively filtered from the blood by the thyroid. After injecting a single dose, the Re\* concentration in that organ is 25 to 100 or more times greater than that found in any other tissue. These findings therefore add support to our hypothesis that the thyroid will selectively filter from the blood all the elements of the seventh periodic group. The greatest concentration of Re\* in tissues occurs within one hour. For several hours it decreases slowly and then more rapidly so that more than 90% is recovered from the urine in 24 hours.

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TABLE III.  
 Cross-complement-fixation Test with Soluble Antigens and Hamster Sera.

Antigen	Dilution	Serum			
		PR8	L <sub>7</sub> 47	L <sub>4</sub> 49	L <sub>5</sub> 49
PR8	1:2	48	12	24	12
	1:4	48	12	24	12
	1:8	24	12	24	24
	1:16	48	12	12	24
	1:32	48	<12	<12	<12
	1:64	12	<12	<12	<12
L <sub>7</sub> 47	1:2	12	24	48	24
	1:4	12	24	24	24
	1:8	12	24	24	24
	1:16	<12	12	<12	<12
	1:32	<12	<12	<12	<12
	1:64	<12	<12	<12	<12
L <sub>4</sub> 49	1:2	12	12	24	24
	1:4	12	12	24	24
	1:8	12	12	24	24
	1:16	<12	12	12	24
	1:32	<12	<12	<12	<12
	1:64	<12	<12	<12	<12
L <sub>5</sub> 49	1:2	12	nt	24	24
	1:4	12	nt	24	24
	1:8	12	nt	24	24
	1:16	<12	nt	12	12
	1:32	<12	nt	<12	<12

Using the complement-fixing allantoic fluid antigen of influenza virus, the 1947 and 1949 strains were distinctly set off from the PR8 virus by the lack of reaction—in the dilutions used—between their sera and the antigen of the PR8 virus (Table II). The anti-PR8 serum with a higher homologous titer than the other antisera reacted with the recent strains. Although when tested with the PR8 antigen in suboptimal dilution (1:16) the homologous and heterologous titers of this serum were the same, with higher concentrations of the PR8 antigen the homologous titer was 4 to 16 times greater than the heterologous titers.

As shown in Table III, the complement-fixation test with the soluble antigens brought out the kinship of these strains. The results with the PR8 antiserum resembled the corresponding findings with the "allantoic fluid" antigen, but the results with the other sera differed from those obtained with the allantoic fluid antigen in that these sera reacted with the PR8 soluble antigen to a titer of about the same magnitude as the homologous titers. This reaction was type-specific. There was

no reaction between any of these sera shown in Tables II and III and the Lee virus antigens.

*Discussion.* The fact that hamster antisera are active in complement fixation as well as hemagglutination tests should make them very useful in the typing of newly isolated strains.

Taylor<sup>6</sup> has employed hamster sera in antigenic analyses of strains of influenza virus. In our laboratory it was possible to type the viruses isolated from the outbreak of influenza of 1949<sup>4</sup> by such a procedure on primary isolation. The membranes of embryos used for primary isolation served as source of the soluble antigen. Once the type of virus has been established, strain differentiation can be accomplished by agglutination-inhibition tests using hamster sera. A general idea about the relationship of the isolated virus to other newly identified strains (subtype specificity) may be obtained from the complement-fixation reaction with al-

<sup>6</sup> Taylor, R. M., *Am. J. Pub. Health*, 1949, 39, 171.

TABLE I.  
Cross-hemagglutination-inhibition Reactions with Hamster Antisera.

Antigen	Serum						
	PR8	FJS	FM <sub>1</sub>	L <sub>3</sub> 47	L <sub>7</sub> 47	L <sub>8</sub> 47	Lee
PR8	2560	20	20	<15	<15	15	<15
FJS	<15	320	20	15	15	<15	<15
FM <sub>1</sub>	<15	60	320	240	80	80	<15
L <sub>3</sub> 47	<15	40	240	320	120	60	<15
L <sub>7</sub> 47	<15	<15	60	60	120	30	<15
L <sub>8</sub> 47	<15	15	30	30	40	240	<15
Lee	<15	<15	<15	<15	<15	<15	80

TABLE II.  
Cross-complement-fixation Test with Allantoic Fluid Antigens and Hamster Sera.

Antigen	Dilution	Serum			
		PR8	L <sub>7</sub> 47	L <sub>4</sub> 49	L <sub>8</sub> 49
PR8	und.	192*	<12	<12	<12
	1:2	192	<12	<12	<12
	1:4	96	<12	<12	<12
	1:8	192	<12	<12	<12
	1:16	24	<12	<12	<12
	1:32	<12	<12	<12	<12
L <sub>7</sub> 47	und.	12	48	24	24
	1:2	12	96	48	24
	1:4	24	96	48	48
	1:8	24	96	48	48
	1:16	12	48	48	24
	1:32	<12	24	<12	<12
L <sub>4</sub> 49	und.	12	48	48	48
	1:2	24	24	48	24
	1:4	24	48	48	48
	1:8	12	48	48	48
	1:16	12	24	24	48
	1:32	<12	12	<12	<12
L <sub>8</sub> 49	und.	12	nt	24	48
	1:2	24	nt	48	48
	1:4	24	nt	48	96
	1:8	12	nt	48	48
	1:16	12	nt	48	24
	1:32	<12	nt	<12	<12

\* Serum titer expressed as the reciprocal of serum dilution.

*Complement Fixation Test.* The procedure of the complement fixation test was as follows: Serial, two-fold dilutions of serum in 0.1 cc amounts were mixed with 0.1 cc amounts of progressively diluted antigen and with 0.2 cc of complement diluted to contain 1.5-2.0 units. The tubes were kept overnight in the refrigerator. The binding of complement was determined by the addition of 0.2 cc of sensitized cells (consisting of 0.1 cc of 2% sheep cells plus 0.1 cc of optimally diluted hemolysin) and incubation at 37°C for 30 minutes. The titers

represent initial dilution of serum, and are based on 3+ or 4+ readings.

*Results.* As shown in Table I, hamster sera gave highly specific reactions in the hemagglutination-inhibition test. Not only was there no reaction between the strains from 1947-1948 and the anti-Lee serum, but there was very little or no cross-relationship between these strains and the PR8 virus. Moreover, the recent strains exhibited antigenic differences among themselves. It appears that the L<sub>3</sub>47 and FM<sub>1</sub> viruses are probably identical strains.

TABLE I. Exp. 1.

Group	% incidence of edema	Degree of edema	% dead	Symptoms
1. Controls	100	3.5+	0	—
2. Adx untreated	80	3.5	10	30% cyanotic and all prostrated
3. Adx + epinephrine	0	0	0	None
4. Adrenal demedullated	80	4.0	0	"

TABLE II. Exp. 2.

Group	% incidence of edema	Degree of edema	% dead	Symptoms
1. Untreated controls	80	4	0	—
2. Adx untreated	100	4	100	Died in 1½-3 hr
3. Adx + epinephrine	50	3	0	Good condition
4. Adrenal demedullated (untreated)	75	4	0	—

histaminic action." Selye<sup>2</sup> and Leger and Masson<sup>3</sup> found that among other things, "alarming stimuli" or stress such as exercise, cold, India ink and formalin injections inhibited the reaction. Since epinephrine protects against egg white edema in the normal rat and against the enhanced histamine sensitivity in the adrenalectomized rat, it seemed possible that it would protect against the increased egg white sensitivity and mortality in adrenalectomized rats as effectively or more so than adrenal cortical extract. Since it was considered possible that "alarming stimuli" may act by releasing endogenous epinephrine, additional experiments were done in adrenal demedullated rats and rats in which adrenergic blockade was induced.

**Methods and Results.** The methods of producing and grading the severity of the edematous symptoms have been described elsewhere.<sup>1</sup> Two to 4 hours after the intraperitoneal injection of fresh, filtered (or redissolved lyophilized) egg white, the edema of the face and extremities was graded by the per cent incidence and by the 0-4+ degree of edema. In addition, where adrenal insufficiency was included, the per cent mortality and symptoms were recorded. Leger and Masson's observations of enhanced sensitivity following adrenalectomy were confirmed. Following adrenalectomy, the rats were kept on the stock diet (Purina Chow) and 1% NaCl + 0.2% NaHCO<sub>3</sub> drinking fluid for

some time before they were given egg white. If the salt therapy was continued up to and following the injection, there were fewer deaths than if they were deprived of salt for 24 hours, but in any case the symptoms were much more severe than in animals with intact adrenals.

**Exp. 1.** Four groups of 10 male rats (Slo-naker strain) each, average body weight 270 g were given egg white, 0.5 cc per rat, intraperitoneally. The operations were performed 10 days prior to injections, and the adrenalectomized rats were kept on salt throughout the experiment. The epinephrine-HCl (Parke, Davis natural product), 0.1 mg/kg was injected subcutaneously at the same time as the egg white. The symptoms were graded 4 hours afterward.

Adrenalectomy enhances the toxicity of the egg white and the severity of the symptoms. Epinephrine affords complete protection, in contrast to adrenal cortical extract.<sup>3</sup> Adrenal demedullation alone does not aggravate the symptoms.

**Exp. 2.** Four groups of 4-5 males each, average body weight 250 g were injected with 1.0 cc egg white per rat. The symptoms were graded 3 hours later. The operations were performed 19 days before the injections, and the adrenalectomized rats were kept on salt until 24 hours before the injections, at which time water was substituted. The epinephrine-HCl, 40 micrograms/kg was injected sub-

lantoic fluid containing virus antigen. Another advantage in using hamsters is the ability of these animals to produce antibodies in response to low concentrations of virus. This makes it possible to prepare antisera to viruses in first or second egg passage. This is not always feasible with chickens because more virus is required to induce antibody formation in these animals. The value of hamster sera is slightly affected by a short-coming; the hemagglutination-inhibition test is difficult to read because of the rapid settling of cells, giving rise to fuzzy or serrated discs of agglutinated cells.

*Summary.* Antisera prepared in hamsters by the intranasal instillation of allantoic fluids containing influenza virus were type-specific when used in the complement-fixation test with the soluble antigens; group- or subtype-specific (e.g. A prime) when used in the complement fixation test with the allantoic fluid antigen, and strain-specific in hemagglutination-inhibition tests.

It appears that the hamster is an animal best suited for quick identification of influenza viruses. It is suggested that their sera be used for systematic classification of strains.

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### Effect of Epinephrine, Antihistaminics and Adrenergic Blockade on Egg White Edema in Adrenal Insufficient Rats.\* (17484)

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Recently we reported a marked protection by 0.1 mg/kg epinephrine hydrochloride against the acute edema induced by the intraperitoneal injection of egg white in rats.<sup>1</sup> Selye<sup>2</sup> and Leger and Masson<sup>3</sup> showed that adrenalectomy greatly sensitized rats to this edema and led to death in a large percentage of the animals. Adrenal cortical extract protected such animals from dying but not from the edema, nor did it confer protection to animals with intact adrenals. Kellaway,<sup>4</sup> Wyman,<sup>5</sup> and Perla and Marmorston-Gottesman,<sup>6</sup> Wy-

man<sup>7</sup> and Ingle, *et al.*<sup>8,9</sup> showed that epinephrine antagonized histamine toxicity in adrenalectomized rats. The egg white edema syndrome could conceivably be caused by the local release of histamine or a histamine-like substance, since antihistaminics effectively combat the edema, as demonstrated by Leger and Masson,<sup>10</sup> Brown and Werner,<sup>11</sup> and ourselves.<sup>1</sup> The doses of antihistaminics required are considerable, however, which may also suggest that the protection results from precapillary arteriolar vasoconstriction<sup>12</sup> and decreased capillary filtration rather than "anti-

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† Published with the approval of the Chief Medical Director, Veterans Administration. The statements and conclusions published by the first author are a result of his own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

<sup>1</sup> Clark, W. G., and MacKay, E. M., *Proc. Soc. Exp. Biol. and Med.*, 1949, **71**, 86.

<sup>2</sup> Selye, H., *Endocrinol.*, 1937, **21**, 169.

<sup>3</sup> Leger, J., and Masson, G. M. C., *Ann. Allergy*, 1949, **0**, 131.

<sup>4</sup> Kellaway, C. H., *J. Physiol.*, 1923, **57**, 82.

<sup>5</sup> Wyman, L. C., *Am. J. Physiol.*, 1928-29, **87**, 29.

<sup>6</sup> Perla, D., and Marmorston-Gottesman, J., *Am. J. Physiol.*, 1929, **89**, 152.

<sup>7</sup> Wyman, L. C., *Am. J. Physiol.*, 1929, **89**, 356.

<sup>8</sup> Ingle, D. J., *Am. J. Physiol.*, 1937, **118**, 57.

<sup>9</sup> Ingle, D. J., Nezamis, J. E., and Kuizenga, M. H., *Exp. Med. and Surg.*, 1947, **5**, 379.

<sup>10</sup> Leger, J., and Masson, G., *Am. J. Med. Sci.*, 1947, **214**, 305.

<sup>11</sup> Brown, B. B., and Werner, H. W., *J. Lab. Clin. Med.*, 1948, **33**, 325.

<sup>12</sup> Haley, T. J., and Harris, H., *J. Pharm. Exp. Therap.*, 1949, **95**, 293.

TABLE I. Exp. 1.

Group	% incidence of edema	Degree of edema	% dead	Symptoms
1. Controls	100	3.5+	0	—
2. Adx untreated	80	3.5	10	30% cyanotic and all prostrated
3. Adx + epinephrine	0	0	0	None
4. Adrenal demedullated	80	4.0	0	"

TABLE II. Exp. 2.

Group	% incidence of edema	Degree of edema	% dead	Symptoms
1. Untreated controls	80	4	0	—
2. Adx untreated	100	4	100	Died in 1½-3 hr
3. Adx + epinephrine	50	3	0	Good condition
4. Adrenal demedullated (untreated)	75	4	0	—

histaminic action." Selye<sup>2</sup> and Leger and Masson<sup>3</sup> found that among other things, "alarming stimuli" or stress such as exercise, cold, India ink and formalin injections inhibited the reaction. Since epinephrine protects against egg white edema in the normal rat and against the enhanced histamine sensitivity in the adrenalectomized rat, it seemed possible that it would protect against the increased egg white sensitivity and mortality in adrenalectomized rats as effectively or more so than adrenal cortical extract. Since it was considered possible that "alarming stimuli" may act by releasing endogenous epinephrine, additional experiments were done in adrenal demedullated rats and rats in which adrenergic blockade was induced.

**Methods and Results.** The methods of producing and grading the severity of the edematous symptoms have been described elsewhere.<sup>1</sup> Two to 4 hours after the intraperitoneal injection of fresh, filtered (or redissolved lyophilized) egg white, the edema of the face and extremities was graded by the per cent incidence and by the 0-4+ degree of edema. In addition, where adrenal insufficiency was included, the per cent mortality and symptoms were recorded. Leger and Masson's observations of enhanced sensitivity following adrenalectomy were confirmed. Following adrenalectomy, the rats were kept on the stock diet (Purina Chow) and 1% NaCl + 0.2% NaHCO<sub>3</sub> drinking fluid for

some time before they were given egg white. If the salt therapy was continued up to and following the injection, there were fewer deaths than if they were deprived of salt for 24 hours, but in any case the symptoms were much more severe than in animals with intact adrenals.

**Exp. 1.** Four groups of 10 male rats (Slo-naker strain) each, average body weight 270 g were given egg white, 0.5 cc per rat, intraperitoneally. The operations were performed 10 days prior to injections, and the adrenalectomized rats were kept on salt throughout the experiment. The epinephrine-HCl (Parke, Davis natural product), 0.1 mg/kg was injected subcutaneously at the same time as the egg white. The symptoms were graded 4 hours afterward.

Adrenalectomy enhances the toxicity of the egg white and the severity of the symptoms. Epinephrine affords complete protection. In contrast to adrenal cortical extract.<sup>3</sup> Adrenal demedullation alone does not aggravate the symptoms.

**Exp. 2.** Four groups of 4-5 males each, average body weight 250 g were injected with 1.0 cc egg white per rat. The symptoms were graded 3 hours later. The operations were performed 19 days before the injections, and the adrenalectomized rats were kept on salt until 24 hours before the injections, at which time water was substituted. The epinephrine-HCl, 40 micrograms/kg was injected sub-



TABLE III. Exp. 3.

Group	% incidence of edema	Degree of edema	% dead	Symptoms
1. Untreated controls	100	4.0	0	—
2. Adx (on salt throughout)	100	4.0	80	Cyanotic and prostrated
3. Adx + epinephrine (on salt throughout)	100	4.0	25	< group 2
4. Adx (off salt 1 day)	80	3.5	80	Cyanotic and prostrated
5. Adx + epinephrine (off salt 1 day)	80	2.5	40	< group 3

TABLE IV. Exp. 4.

Group	% incidence of edema	Degree of edema	% dead	Symptoms
1. Untreated controls	100	4.0+	0	—
2. " " + SY-28	0		0	None
3. Adx untreated	100	4.0	80	Cyanotic and prostrated
4. Adx untreated + SY-28	0		0	Prostrated

cutaneously at the same time as the egg white (which is a sub-effective edema-preventative dose).<sup>1</sup>

Doses of epinephrine which fail to prevent egg white edema even in animals with intact adrenals, completely protect against the fatal effects of egg white intoxication which kills 100% of the untreated adrenalectomized rats. Adrenal demedullation alone does not enhance the edema.

*Exp. 3.* Five groups of 5 males each, average body weight 180 g, were given egg white 0.5 cc per rat. The symptoms were graded 2 hours later. The adrenalectomies were performed 14 days before the injections, and the animals were maintained on salt both throughout the experiment, and withheld 24 hours prior. The epinephrine-HCl, 0.2 mg/kg was injected subcutaneously at the same time as the egg white injections.

As in Exp. 2 vs 1, intensification of adrenal insufficiency by salt deprivation enhances the susceptibility of adrenalectomized rats, but epinephrine still exerts its protection, in confirmation of the previous two experiments.

*Exp. 4.* Four groups of 5 males each, average body weight 180 g were given egg white 0.5 cc per rat. The symptoms were graded 2 hours later. The adrenalectomies were performed 14 days before the injections, and the rats were maintained on salt through-

out the experiment. "SY-28", a  $\beta$ -haloalkyl amine (dibenamine congener) adrenergic blocking drug, N-(2-bromoethyl)-1-naphthalene-methylamine hydrobromide,<sup>†</sup> was given 10 mg/kg subcutaneously at the same time as egg white.

SY-28 in the dose employed, protects normal and adrenalectomized rats against edema and death, although it was by itself toxic to adrenalectomized rats. "SY-28" is a potent antihistaminic<sup>13,15</sup> in addition to being a very effective adrenergic blocking drug,<sup>13,18</sup> as are several other  $\beta$ -haloalkylamines.<sup>13-15,17,19</sup> Since it is known that antihistaminics inhibit egg white edema in rats with intact adrenals,<sup>1,10,11</sup> it was felt that experiments

<sup>†</sup> Generously supplied by Dr. G. Rieveschl, Jr., Parke, Davis and Co., Detroit, Mich.

<sup>13</sup> Loew, E. R., and Mietich, A., *J. Pharm. Exp. Therap.*, 1948, **94**, 339.

<sup>14</sup> Stone, C. A., and Loew, E. R., *J. Pharm. Exp. Therap.*, 1948, **94**, 350.

<sup>15</sup> Vleeschhouwer, G. R. de, *Proc. Soc. Exp. Biol. and Med.*, 1948, **67**, 298.

<sup>16</sup> Nickerson, M., and Gump, W. S., *J. Pharm. Exp. Therap.*, 1949, **97**, 25.

<sup>17</sup> Nickerson, M., and Harris, F. B., *Fed. Proc.*, 1949, **8**, 321.

<sup>18</sup> Nickerson, M., *J. Pharm. Exp. Therap.*, 1949, **95**, Part II, 27.

<sup>19</sup> Loew, E. R., and Mietich, A., *J. Pharm. Exp. Therap.*, 1949, **95**, 448.

TABLE V. Exp. 5.

Group	% incidence of edema	Degree of edema	% dead	Symptoms
1 cc egg white				
Adx saline controls	100	2.0	100	Severe cyanosis
" phenergan, 5 mg/kg	60	1.5	20	Cyanosis
" " 10 mg/kg	100	2.0	20	Some cyanosis
" " 25 mg/kg	60	1.0	0	None
No egg white				
Adx saline controls	—	—	0	"
" phenergan, 5 mg/kg	—	—	0	"
" " 10 mg/kg	—	—	0	"
" " 25 mg/kg	—	—	0	"

should be performed in which an antihistaminic as well as a purely adrenergic blocking agent was used in adrenal insufficient animals. As an antihistaminic, Phenergan<sup>§</sup> (N-dimethylamino-2-propyl-1-thiodiphenylamine, "32 77-RP") was selected because of its potent antihistaminic action and its anti-capillary permeability effects,<sup>12,20-30</sup> and because in the previous report<sup>1</sup> it was found to be a highly effective inhibitor of egg white edema in the normal rat.

Exp. 5. Eight groups of 5 males each were adrenalectomized 8-10 days prior to the experiment and maintained on salt until 24 hr prior to the experiment, at which time water was substituted. The average body weight at

the time of the experiment was 175 g. Phenergan in 2 cc volume was given subcutaneously to the first 4 groups in doses of 5, 10 and 25 mg/kg simultaneously with 1 cc egg white intraperitoneally, and in similar doses to the last 4 groups without egg white, in order to see if the doses of drug given in the absence of egg white were toxic in adrenal insufficient rats. The symptoms were graded 2 hours later.

Phenergan, a potent antihistaminic and anti-capillary "permeability" drug, effectively antagonizes egg white toxicity in adrenal in-

TABLE VI. Exp. 6.

Group (normal rats)	% incidence of edema	Degree of edema
1. Egg white, 1 cc/kg	0	0
2. " " 2 "	0	0
3. " " 3 "	0	0
4. " " 4 "	30	2.0+

§ In order to rule out the possibility that Phenergan protected normal rats against egg white edema, as previously reported,<sup>1</sup> via epinephrine release from the adrenal medulla, experiments were performed on 10 normal and 10 adrenal demedullated rats given 2 cc/kg egg white intraperitoneally, and with and without 2.5 mg/kg Phenergan subcutaneously 1 hour prior to the egg white. There were no differences in the incidence, onset and intensity of the resulting severe edema, in confirmation of our previous work in which it was found that 10 mg/kg was inhibitory.<sup>1</sup> In another experiment on 20 adrenal demedullated rats, however, 25 mg/kg of Phenergan completely inhibited the edema, whereas the untreated animals had marked edema. This experiment therefore rules out the possibility, suggested by the nearly convulsive doses of antihistaminics used by Leger and Masson,<sup>10</sup> and Brown and Werner,<sup>11</sup> that the antihistaminics act by releasing epinephrine from the adrenal glands.

§ Generously supplied by Drs. A. Gibson and R. C. Pogge of Merck and Co., Inc., Rahway, N. J.

<sup>20</sup> Halpern, B. N., *J. Allergy*, 1947, **18**, 263.

<sup>21</sup> Halpern, B. N., and Cruchard, S., *Compt. rend. Soc. de biol.*, 1947, **141**, 1038.

<sup>22</sup> Halpern, B. N., *Acta Allergologica*, 1948, **1**, 3.

<sup>23</sup> Halpern, B. N., Hamburger, F., and Cruchard, S., *Acta Allergologica*, 1948, **1**, 97.

<sup>24</sup> Halpern, B. N., Guillaumat, L., and Cruchard, S., *Acta Allergologica*, 1948, **1**, 376.

<sup>25</sup> Hamburger, J., Halpern, B. N., and Neel, J., *Compt. rend. Soc. de biol.*, 1948, **142**, 183.

<sup>26</sup> Halpern, B. N., Guillaumat, L., and Cruchard, S., *Compt. rend. Soc. de biol.*, 1948, **142**, 622.

<sup>27</sup> Halpern, B. N., Vermeil, G., and Cruchard, S., *Compt. rend. Soc. de biol.*, 1948, **142**, 1385.

<sup>28</sup> Halpern, B. N., and Laubseher, J., *Sem. Hop.*, 1948, **24**, 667.

<sup>29</sup> Halpern, B. N., and Hamburger, J., *Canad. Med. Assn. J.*, 1948, **59**, 322.

<sup>30</sup> Reuse, J. J., *Compt. rend. Soc. de biol.*, 1948, **142**, 638.

<sup>31</sup> Kerwin, J., Ulyot, G. E., Fellows, E. J., and Macko, E., *Fed. Proc.*, 1949, **8**, 308.

TABLE VII. Exp. 7.

Group	Egg white, cc/kg	% incidence of edema	Degree of edema	% dead
1. Normal controls	2	100	3+	0
2. " "	4	100	3	0
3. Normal, + SKF-501	2	83	2	0
4. " "	4	83	2	0
5. Adx controls	2	83	2	33
6. " "	4	83	2	83
7. " + SKF-501	2	100	2	17
8. " + " "	4	83	2	33

sufficient rats<sup>||</sup> and does so in doses which are not toxic in adrenalectomized rats not given egg white. As an adrenergic blocking agent without antihistaminic action, "SKF-501,"<sup>¶</sup> N-(9-fluorenyl)-N-ethyl- $\beta$ -chloroethylamine, was chosen since it is an even more powerful yet less toxic adrenergic blocking drug than Dibenamine itself,<sup>21</sup> but has little or no antihistaminic activity,\*\* as might be predicted on the basis that in over 100 compounds tested by Nickerson,<sup>16-18</sup> only  $\alpha$ -naphthylmethyl- and  $\beta$ -phenoxyethyl derivatives of the  $\beta$ -haloalkylamines were found to be effective antihistaminics.

*Exp. 6.* Four groups of 3 normal male rats each, average body weight 310 g were given egg white, 1, 2, 3 and 4 cc/kg, respectively, of 1:1 egg white-saline mixture intraperitoneally 3 hours after 2 mg/kg SKF-501 intravenously. The symptoms were graded 2 and 3 hours later.

SKF-501, a purely adrenergic blocking agent, inhibits egg white edema in normal rats.

*Exp. 7.* Forty-eight male rats were divided into 8 groups of 6 each, with an average body weight of 310 g. The operated groups were adrenalectomized 7 days prior to the experiment and were maintained on salt until 24 hours prior to the experiment, at which time water was substituted. The drug-treated groups were given 2 mg/kg SKF-501 intravenously 1 hour before egg white. The egg white was given in 2 doses, 2 cc/kg and 4 cc/kg intraperitoneally, and the symptoms

were graded 3 hours later.

SKF-501, a purely adrenergic blocking drug, decreases the mortality caused by egg white edema in adrenal insufficient rats. The protection in normal controls is somewhat less when given 1 hour before than when given 3 hours before as in Exp. 6.

*Discussion.* The edema and toxicity induced in normal and adrenal insufficient rats by the intraperitoneal injection of egg white is inhibited by epinephrine and arterenol and probably other pressor substances, but not by Isuprel,<sup>††</sup> 1-(3',4'-dihydroxyphenyl)-2-isopropylaminoethanol-HCl,<sup>1</sup> (*present paper*). The cephalic edema caused by *p*-phenylenediamine is inhibited by epinephrine, tyramine, phenylethanolamine, drugs which release endogenous epinephrine such as strychnine and nicotine, and by sympathetic stimulation.<sup>32-36</sup> Epinephrine also relieves the edema associated with urticaria and angioneurotic edema. In adrenal insufficiency the increased capillary filtration of fluids induced by traumatic shock, intraperitoneal administration of hypertonic solutions, egg white, and in all probability *p*-phenylenediamine, intravenous hyaluronidase<sup>37</sup> and other conditions, probably all lead to hypotensive shock as a result of an un-

†† Not investigated in adrenalectomized rats.

<sup>32</sup> Hanzlik, P. J., *J. Ind. Hyg.*, 1923, 4, 386.

<sup>33</sup> Tainter, M. L., and Hanzlik, P. J., *J. Pharm. Exp. Therap.*, 1924, 24, 179.

<sup>34</sup> Gibbs, O. S., *J. Pharm. Exp. Therap.*, 1923, 20, 221; 1931, 42, 65.

<sup>35</sup> Tainter, M. L., *J. Pharm. Exp. Therap.*, 1926, 27, 201; 1928, 33, 129.

<sup>36</sup> Cohen, M. B., Wasserman, P., and Rudolph, J. A., *J. Pharm. Exp. Therap.*, 1933, 48, 235.

<sup>37</sup> Elster, S. K., Freeman, M. E., and Dorfman, A., *Am. J. Physiol.*, 1949, 156, 429.

¶ Generously supplied by Dr. Glen E. Ulyot of Smith, Kline and French Laboratories, Philadelphia, Pa.

\*\* As tested by the prevention of histamine-induced spasm of the isolated guinea pig intestine.<sup>16</sup>

compensated decreased blood volume. Epinephrine may antagonize all of those effects by its arteriolar vasoconstrictive action; and in addition protects adrenalectomized animals against histamine and anaphylactic shock. Antihistaminics inhibit egg white edema, hyaluronidase edema,<sup>38</sup> urticarias, angioneurotic edema and histamine and anaphylactic shock probably both by antagonizing the action of released histamine and by decreasing capillary filtration by arteriolar vasoconstriction. No explanation can be given for the protective effect of adrenergic blockade in egg white edema. At first thought, it might have been anticipated that it would aggravate the condition by blocking the protective action of endogenous epinephrine and sympathin-N. This is not the case, however, and the fact the adrenal demedullation also is not detrimental is confirmatory of this lack of effect. It is possible that SY-28 protect in the same way that Dibenamine does in hemorrhagic and traumatic shock, as demonstrated by Remington,<sup>39,40</sup> although the mode of action in both cases is unknown. The effect suggests, however, the participation of an adrenergic component in egg white edema, as seems to be the

case in the pulmonary edemas induced by ammonium chloride<sup>41,42</sup> and increased intracranial pressure or brain concussion.<sup>43</sup>

**Summary.** Epinephrine protects adrenal insufficient rats from the edema and mortality caused by the intraperitoneal administration of egg white. Mortality is prevented by doses (0.04 mg/kg) which are not able to prevent edema in rats with intact adrenals.

The antihistaminic Phenergan, N-dimethylamino-2-propyl-1-thiodiphenylamine ("3277 RP"), in doses as low as 5 mg/kg prevent death from egg white edema in adrenalectomized rats.

"SY-28", N-(2-bromoethyl)-1-naphthalene-methylamine hydrobromide, a Dibenamine congener possessing both antihistaminic and adrenergic blocking activity, also prevents the edema and death, but is in itself toxic to adrenalectomized rats.

"SKF-501", N-(9-fluorenyl)-N-ethyl-β-chloroethylamine, a Dibenamine congener with potent adrenergic blocking powers but with a low toxicity and without antihistamine action, also inhibits the edema and decreases the mortality.

The possible modes of action are discussed.

<sup>41</sup> Koenig, H., and Koenig, R., *Am. J. Physiol.*, 1949, **158**, 1.

<sup>42</sup> MacKay, E. M., Jordan, M., and MacKay, L. L., *Proc. Soc. Exp. Biol. and Med.*, 1949, **72**, 421.

<sup>43</sup> MacKay, E. M., in press.

Received October 18, 1949. P.S.E.B.M., 1949, **72**.

<sup>38</sup> Elster, S. K., Freeman, M. E., and Lowry, E. L., *J. Pharm. Exp. Therap.*, 1949, **96**, 332.

<sup>39</sup> Remington, J. W., Wheeler, N. C., Boyd, G. H., Jr., and Caddell, H. M., *Proc. Soc. Exp. Biol. and Med.*, 1948, **69**, 146.

<sup>40</sup> Remington, J. W., *Fed. Proc.*, 1949, **8**, 131.

## A Microbiological Assay for Vitamin B<sub>12</sub> Using *Lactobacillus leichmannii*.<sup>\*</sup> (17485)

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*Lactobacillus leichmannii* (ATCC 4797) has been reported by several laboratories<sup>1-3</sup> to be a useful test organism for the microbiological measurement of vitamin B<sub>12</sub>. Skeggs, *et al.*<sup>1</sup> found ascorbic acid and air to stimulate a growth response in this organism in the absence of vitamin B<sub>12</sub> on a basal

medium containing casein hydrolysates, but were able to minimize these effects by autoclaving the test assay for 15 minutes at 120°C. Other workers<sup>3</sup> also found certain reducing agents to promote a growth response in both *L. leichmannii* (ATCC 4797) and *L. leichmannii* (ATCC 7830) in the absence

of vitamin B<sub>12</sub> on casein hydrolysate media, but in addition found that these agents were able to increase the growth response produced by vitamin B<sub>12</sub>. In attempting to overcome these difficulties, a microbiological assay has been developed which is highly sensitive to vitamin B<sub>12</sub> and which yields consistent results. The experimental basis and the details of the procedure are presented in this report.

**Assay Procedure.** The culture of *Lactobacillus leichmannii* 4797 used in this assay was obtained from the American Type Culture Collection, Georgetown University, Washington, D. C. *L. leichmannii* (ATCC 7830) has also been used successfully in this assay. The culture is maintained in a stab medium composed of 5 ml of double strength basal medium (Table I), 0.3 unit of 15 unit USP injectable liver extract, 150 mg of agar and distilled water to make 10 ml. Each week it is transferred to a broth medium<sup>4</sup> in which it is grown for 24 hours at 37°C and is then taken back to the stab medium. The inoculum is prepared by growing the organism for 24 hours in a broth medium having the same composition as the stab medium except for the omission of agar. The procedure for the inoculation and incubation of the assay is the same as that described by Daniel, *et al.*<sup>5</sup> except that the diluted inoculum is

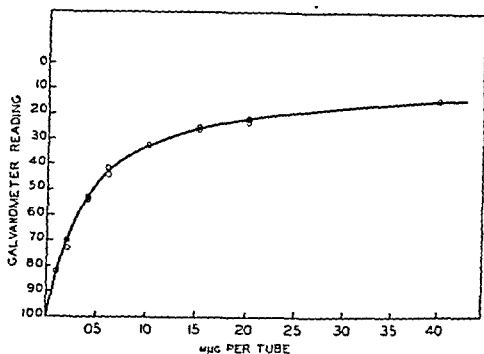


FIG. 1.  
Response of *Lactobacillus leichmannii* to crystalline vit. B<sub>12</sub>.

made to read 60 on the galvanometer scale of a Coleman spectrophotometer at wave length 650 mμ. The composition of the amino acid medium used in this method is presented in Table I. It is a modification of the medium used by Daniel, *et al.*<sup>5</sup> in studies on the nutrition of *L. casei* and that of Snell, *et al.*<sup>6</sup> for *L. leichmannii*.

After all constituents of the medium are combined, the pH is adjusted to 5.5 with 20% potassium hydroxide. The volume is then made to 1 liter and the medium steamed for 5 minutes. Five ml of the double-strength basal medium and the sample to be assayed are placed in the assay tubes. The final volume is made to 10 ml. The assay is incubated at 37°C for 16 hours or until the tube containing 0.1 mγ of vitamin B<sub>12</sub> reaches a turbidimetric reading of approximately 30 on the galvanometer scale of the spectrophotometer. At this time the turbidity of the cell suspension is read by placing the inoculated blanks at 100 on the galvanometer scale. Very little growth, if any, is ordinarily present in the blanks. A typical standard curve is shown in Fig. 1. The results show that on this basal medium the assay range is between 0.001 and 0.04 mγ of vitamin B<sub>12</sub> per ml of medium.

The canned tomato juice used in this method is purchased on the open market. It is filtered through No. 617 Eaton and Dikeman<sup>†</sup>

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2 Capps, B. F., Hobbs, N. L., and Fox, S. H., *J. Biol. Chem.*, 1949, **178**, 517.

3 Stokstad, E. L. R., Dornbush, A. C., Franklin, A. L., Hoffman, C. E., Hutchings, B. L., and Jukes, T. H., *Fed. Proc.*, 1949, **8**, 257.

4 Nymon, M. C., Gunsalus, I. C., and Gortner, W. A., *Science*, 1945, **102**, 125.

5 Daniel, L. J., Scott, M. L., Heuser, G. F., and Norris, L. C., *J. Biol. Chem.*, 1948, **174**, 71.

6 Snell, E. E., Kitay, E., and McNutt, W. S., *J. Biol. Chem.*, 1948, **175**, 473.

filter paper on a Büchner funnel with the aid of Celite filter aid (Johns-Manville). The resulting solution is adsorbed with 12.5 g of activated norit per liter at the natural pH of the serum (4.0 to 4.2) for ½ hour and filtered to remove the norit.

*Development of Assay.* Crystalline vitamin B<sub>12</sub><sup>†</sup> and a potent vitamin B<sub>12</sub> concentrate<sup>‡</sup> were used in the development of this assay. The vitamin B<sub>12</sub> concentrate was prepared from 15 unit USP injectable liver extract by the technic of counter-current distribution as described by Craig.<sup>6</sup> The results of a study showing the need of *L. leichmannii* for a growth factor present in tomato juice is presented in Table II. Crystalline vitamin B<sub>12</sub> was observed to produce a submaximal plateau response when assayed on the amino acid medium containing no tomato juice. The addition of adsorbed tomato juice filtrate promoted a much improved growth response from the vitamin especially at the higher levels. Increasing the content of ferrous sulfate and cysteine, or adding ascorbic acid or sodium thioglycollate to the medium, did not replace the need for the supplement contrary to the findings of Stokstad, *et al.*<sup>3</sup> The same results were obtained when lyophilized tomato juice filtrate was employed.

Preliminary experiments showed also that a phenol-n-butyl alcohol extract of USP injectable liver extract which had been freed of vitamin B<sub>12</sub> and thymidine is capable of replacing tomato juice as a source of unknown factors in the assay medium. Other studies indicated that the factor for *L. leichmannii* and that reported by Daniel, *et al.*<sup>5,9</sup> for *L. casei* are concentrated by counter-current distribution in the same fractions of injectable liver extracts and therefore may be identical. Whether this factor is the same as that reported by Shorb<sup>10</sup> has not been determined.

During some early studies it was found that

TABLE I.  
Double-Strength Medium\* for Assay of Vit. B<sub>12</sub>  
Using *Lactobacillus leichmannii*.

DL-Alanine	425	mg
L-Arginine	425	"
DL-Aspartic acid	425	"
L-Cystine	400	"
L-Glutamic acid	1213	"
Glycine	425	"
L-Histidine	425	"
L-Hydroxyproline	50	"
DL-Isoleucine	425	"
DL-Leucine	425	"
L-Lysine	213	"
DL-Methionine	425	"
DL-Norleucine	425	"
DL-Phenylalanine	100	"
L-Proline	425	"
DL-Serine	50	"
DL-Threonine	50	"
DL-Tryptophan	50	"
L-Tyrosine	400	"
DL-Valine	425	"
Asparagine	150	"
Glutamine	200	"
Glucose	40	g
Cysteine hydrochloride	2	"
Sodium acetate (trihydrate)	12	"
Sodium citrate	10	"
MgSO <sub>4</sub> · 7H <sub>2</sub> O	2.8	"
FeSO <sub>4</sub> · 7H <sub>2</sub> O	800	mg
MnSO <sub>4</sub> · 4H <sub>2</sub> O	600	"
KH <sub>2</sub> PO <sub>4</sub>	4	g
K <sub>2</sub> HPO <sub>4</sub>	4	"
Adenine sulfate	10	mg
Guanine hydrochloride	10	"
Uracil	10	"
Xanthine	15	"
Tween 80†	2	ml
Tomato juice filtrate‡	600	"
Pyridoxine	4	mg
Pyridoxal	4	"
Niacin	2	"
Riboflavin	2	"
Thiamine	2	"
Calcium pantothenate	2	"
Biotin	10	γ
Folic acid	110	"
Pyridoxamine	800	"
p-Aminobenzoic acid	80	"
Distilled water to make	1000	ml

\* After preparation the pH of the medium is adjusted to 5.5 with 20% potassium hydroxide, steamed 5 minutes and cooled.

† Polyoxyethylene sorbitan monooleate (Atlas Powder Co.).

‡ Prepared as described previously.

† The Eaton-Dikeman Company, Mt. Holly Springs, Pa.

‡ The crystalline vitamin B<sub>12</sub> used in this work was kindly supplied by Dr. E. Lester Smith of Glaxo Laboratories, England, and Dr. D. F. Green of Merck and Co., Rahway, N. J.

7 Carlson, C. W., Thesis, Cornell University.

8 Craig, L. C., *J. Biol. Chem.*, 1944, 155, 519.

better growth is obtained with *L. leichmannii* at the same level of vitamin B<sub>12</sub> when the initial pH of the medium was more acidic than the usual value of 6.6 to 6.8.<sup>1,2,6</sup> In

9 Daniel, L. J., Peeler, H. T., Norris, L. C., and Scott, M. L., *J. Biol. Chem.*, 1949, 177, 917.

10 Shorb, Mary S., *J. Bact.*, 1947, 53, 669.

of vitamin B<sub>12</sub> on casein hydrolysate media, but in addition found that these agents were able to increase the growth response produced by vitamin B<sub>12</sub>. In attempting to overcome these difficulties, a microbiological assay has been developed which is highly sensitive to vitamin B<sub>12</sub> and which yields consistent results. The experimental basis and the details of the procedure are presented in this report.

**Assay Procedure.** The culture of *Lactobacillus leichmannii* 4797 used in this assay was obtained from the American Type Culture Collection, Georgetown University, Washington, D. C. *L. leichmannii* (ATCC 7830) has also been used successfully in this assay. The culture is maintained in a stab medium composed of 5 ml of double strength basal medium (Table I), 0.3 unit of 15 unit USP injectable liver extract, 150 mg of agar and distilled water to make 10 ml. Each week it is transferred to a broth medium<sup>4</sup> in which it is grown for 24 hours at 37°C and is then taken back to the stab medium. The inoculum is prepared by growing the organism for 24 hours in a broth medium having the same composition as the stab medium except for the omission of agar. The procedure for the inoculation and incubation of the assay is the same as that described by Daniel, *et al.*<sup>5</sup> except that the diluted inoculum is

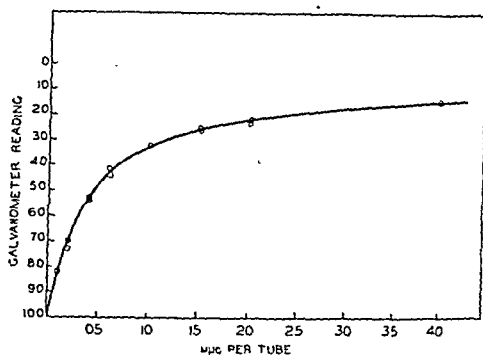


FIG. 1.  
Response of *Lactobacillus leichmannii* to crystalline vit. B<sub>12</sub>.

made to read 60 on the galvanometer scale of a Coleman spectrophotometer at wave length 650 mμ. The composition of the amino acid medium used in this method is presented in Table I. It is a modification of the medium used by Daniel, *et al.*<sup>5</sup> in studies on the nutrition of *L. casei* and that of Snell, *et al.*<sup>6</sup> for *L. leichmannii*.

After all constituents of the medium are combined, the pH is adjusted to 5.5 with 20% potassium hydroxide. The volume is then made to 1 liter and the medium steamed for 5 minutes. Five ml of the double-strength basal medium and the sample to be assayed are placed in the assay tubes. The final volume is made to 10 ml. The assay is incubated at 37°C for 16 hours or until the tube containing 0.1 mγ of vitamin B<sub>12</sub> reaches a turbidimetric reading of approximately 30 on the galvanometer scale of the spectrophotometer. At this time the turbidity of the cell suspension is read by placing the inoculated blanks at 100 on the galvanometer scale. Very little growth, if any, is ordinarily present in the blanks. A typical standard curve is shown in Fig. 1. The results show that on this basal medium the assay range is between 0.001 and 0.04 mγ of vitamin B<sub>12</sub> per ml of medium.

The canned tomato juice used in this method is purchased on the open market. It is filtered through No. 617 Eaton and Dikeman<sup>†</sup>

\* This work was undertaken in cooperation with the Office of Naval Research, Navy Department, Washington, D.C., and was aided by grants to Cornell University by the Nutrition Foundation, Inc., New York, the Cerophyl Laboratories, Inc., Kansas City, Mo., The Commercial Solvents Corporation, New York City, The Cooperative G.L.F. Exchange, Ithaca, N. Y., and the Western Condensing Company, San Francisco, Calif. This work was conducted in the Nutrition Laboratories of the Department of Poultry Husbandry. The technical assistance of Betty F. Brown and Diana M. Cameron is acknowledged.

<sup>1</sup> Skeggs, H. R., Huff, J. W., Wright, L. D., and Bosshardt, D. K., *J. Biol. Chem.*, 1948, **170**, 1459.

<sup>2</sup> Capps, B. F., Hobbs, N. L., and Fox, S. H., *J. Biol. Chem.*, 1949, **178**, 517.

<sup>3</sup> Stokstad, E. L. R., Dornbush, A. C., Franklin, A. L., Hoffman, C. E., Hutchings, B. L., and Jukes, T. H., *Fed. Proc.*, 1949, **8**, 257.

<sup>4</sup> Nymon, M. C., Gunsalus, I. C., and Gortner, W. A., *Science*, 1945, **102**, 125.

<sup>5</sup> Daniel, L. J., Scott, M. L., Heuser, G. F., and Norris, L. C., *J. Biol. Chem.*, 1948, **174**, 71.

<sup>6</sup> Snell, E. E., Kitay, E., and McNutt, W. S., *J. Biol. Chem.*, 1948, **175**, 473.

TABLE II.  
Response of *L. leichmannii* to Vit. B<sub>12</sub> in Presence and Absence of an Unidentified Growth Factor in the Medium.

Substance	Level per tube mγ	Galvanometer readings*	
		Medium without tomato juice filtrate	Medium with tomato juice filtrate
None		100	100
Crystalline vit. B <sub>12</sub>	.012	85	80
	.036	75	57
	.059	66	42
	.118	57	26
	.236	50	20
	.412	53	13

\* A galvanometer reading of 100 represents no growth.

TABLE III.  
Comparison of Two Different Media in the Assay of Vit. B<sub>12</sub>.

Substance	Level per tube, mγ	Galvanometer readings*	
		Amino acid medium†	Casein-hydrolysate medium‡
None		100	100
Crystalline vit. B <sub>12</sub>	.012	76	95
	.036	49	89
	.059	35	79
	.118	26	61
	.236	16	40
	.412	13	24
Vit. B <sub>12</sub> conc.§	.012	74	98
	.036	50	93
	.059	36	90
	.118	23	93
	.236	15	83
	.412	11	74
Adsorbed enzyme digested casein	mg 25	18	—

\* A galvanometer reading of 100 means no growth.

† The amino acid medium reported in this paper.

‡ Prepared as described by Skeggs *et al.*, *J. Biol. Chem.*, 1948, **170**, 1459.

§ Prepared as described previously.

|| A constituent of the casein-hydrolysate medium. Adsorbed three times the amount reported necessary by Skeggs *et al.*, *J. Biol. Chem.*, 1948, **170**, 1459.

The results of studies on the recovery of vitamin B<sub>12</sub> from USP injectable liver extract are presented in Table IV. The results showed a mean recovery of 100.8% from this product. These results were confirmed by conducting recoveries from Wilson's liver fraction L. In this case individual recoveries ranged from 96.7 to 109.4% with a mean recovery of 100.7%. No synergism was found to exist between the natural materials and crystalline vitamin B<sub>12</sub>. The assay method,

therefore, appears to be specific for vitamin B<sub>12</sub> activity. Further evidence of this was obtained in counter-current distribution studies of injectable liver extract since the assay of the fractions in the various tubes indicated the partition of a single substance.

Thymidine,§ which was shown to promote

§ The thymidine used in these studies was kindly supplied by Dr. William Shive of the University of Texas.



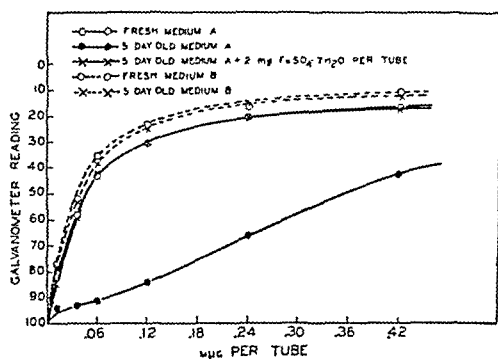


FIG. 2.

Response of *Lactobacillus leichmannii* to vit. B<sub>12</sub> concentrate on fresh and aged media. Medium A contains no cysteine and only 1.11 mg per tube of FeSO<sub>4</sub>·7H<sub>2</sub>O. Medium B contains 4 mg per tube of FeSO<sub>4</sub>·7H<sub>2</sub>O and 10 mg per tube of cysteine.

view of this finding, studies were undertaken to establish the optimum pH range for this organism on the amino acid medium. Nine samples of the medium were used. Each contained vitamin B<sub>12</sub> in such quantity that, on dispensing, every assay tube had 0.4 my of vitamin B<sub>12</sub>. The pH of these samples was adjusted so as to have a graded series ranging from 4.0 to 7.0. The results showed that under these conditions maximum growth was progressively retarded at pH values greater, or less, than pH 5.5.

On a medium prepared as shown in Table I, but containing no cysteine and only 222 mg of FeSO<sub>4</sub>·7H<sub>2</sub>O per liter, poorer growth was obtained at the same level of vitamin B<sub>12</sub> when the medium was a few days old than when it was prepared fresh. This effect became marked if a vitamin B<sub>12</sub> concentrate prepared from a 15 unit USP injectable liver extract was used as the supplement. In attempting to overcome the effect of aging, the addition of 2 mg of ferrous sulphate per tube to the aged medium was found to restore it to a "fresh" condition. Response curves demonstrating this are shown in Fig. 2.

Studies were then undertaken to determine how to preserve the medium in a fresh condition for longer periods of time. In these experiments 0.118 my of vitamin B<sub>12</sub> concentrate per tube was assayed in the presence of graded levels of FeSO<sub>4</sub>·7H<sub>2</sub>O and cysteine. The results of the study showed that the

growth response reached a plateau with either 4 mg of ferrous sulfate, or 15 mg of cysteine per tube. A combination of 4 mg of ferrous sulfate per tube and graded levels of cysteine, however, promoted an additional growth response which reached a plateau with only 4 mg of cysteine per tube. Additional cysteine had no effect on growth but aided in maintaining the medium in a fresh state for a longer period of time. As a consequence of these findings, the composition of the medium was changed so that it contains 4 mg of FeSO<sub>4</sub>·7H<sub>2</sub>O and 10 mg of cysteine per tube. The effect of allowing the medium containing the increased iron and cysteine to age 5 days before use is also shown in Fig. 2.

Certain discrepancies were found to exist between the assays conducted on the amino acid medium described in this report and those conducted on a medium prepared as described by Skeggs, *et al.*<sup>1</sup> The responses of crystalline vitamin B<sub>12</sub> and a vitamin B<sub>12</sub> concentrate using these media are presented in Table III. The values from the amino acid medium were obtained at 16 hours and those on the casein hydrolysate medium at 24 hours.<sup>1</sup> In each case more growth resulted from the same level of vitamin B<sub>12</sub> on the amino acid medium than on the other. Furthermore, the vitamin B<sub>12</sub> concentrate which was shown to be highly active for the chick<sup>11</sup> promoted only slight response on the casein hydrolysate basal medium. On investigating the cause of this discrepancy it was found that addition of ferrous sulfate to the casein hydrolysate medium markedly increased the growth response from the vitamin B<sub>12</sub> concentrate which is in agreement with the work of Stokstad, *et al.*<sup>3</sup> The assay blanks were found to have considerable growth under these conditions. This growth was found to be due to the hydrolyzed casein in the medium and indicated that casein hydrolysate contained a form of vitamin B<sub>12</sub> active only in the presence of reducing agents. This is probably the same effect noted by Welch, *et al.*<sup>12</sup>

<sup>11</sup> Carlson, C. W., Miller, R. F., Peeler, H. T., Norris, L. C., and Heuser, G. F., *Poultry Sci.*, 1949, **28**, 750.

<sup>12</sup> Welch, A. D., and Wilson, F. F., *Arch. Biochem.*, 1949, **22**, 486.

TABLE VI.  
Vit. B<sub>12</sub> Content of Several Materials.

Material	Vit. B <sub>12</sub> content, mγ/g
Soybean meal	11
Yellow corn	10
Wheat	7
Alfalfa leaf meal	40
Dried brewers' yeast	8
Wilson's liver L	389
Crude casein	104
White fish meal	98.3
Red fish meal	111
Condensed fish solubles	92
Meat scraps	42.3

takadiastase is added to the mixture together with 2 mg of neutralized cysteine hydrochloride and 1 ml of toluene. The materials are well mixed and incubated for 40 hours at 37°C. After incubation the samples are adjusted to pH 5.5, steamed 5 minutes, cooled, adjusted to 200 ml and filtered through Whatman fluted filter paper No. 12.

Other treatments used in an attempt to liberate the vitamin include autoclaving at

acidic and neutral pH, allowing the samples to autolyze for 20 hours in acetate buffer, steaming 30 minutes in acetate buffer and allowing the sample to stand in distilled water. These treatments did not liberate as much vitamin B<sub>12</sub> as was obtained by the methods outlined in detail. Treatment of the animal products with trypsin or pepsin produced the same results as were obtained with pancreatin. However, pancreatin is used in the procedure because less growth occurred in the enzyme assay blanks.

*Summary.* A microbiological assay for vitamin B<sub>12</sub> using *Lactobacillus leichmannii* (ATCC 4797) has been developed. The assay medium contains crystalline amino acids as the nitrogen source and adsorbed tomato juice filtrate as a source of unidentified growth factors. A high level of cysteine and ferrous sulfate are present in the medium to keep it in the necessary reduced state. Data are given to show the degree of precision and reproducibility to be expected from the assay.

Received Sept. 3, 1949. P.S.E.B.M., 1949, 72.

TABLE IV.  
 Recoveries of Crystalline Vit. B<sub>12</sub> Added to Liver Fractions.

Substance	Level per tube, units	Vit. B <sub>12</sub> by assay, m $\gamma$	Added B <sub>12</sub> per tube, m $\gamma$	Theoretical B <sub>12</sub> per tube, m $\gamma$	Actual B <sub>12</sub> per tube, m $\gamma$	Recovery, %	Standard error of the mean
USP injectable	.000012	.008	.012	.020	.021	105.0	
liver extr.	.000025	.012	.012	.024	.026	108.3	
(15 unit)	.00005	.025	.012	.037	.035	94.6	
	.000075	.037	.012	.049	.051	104.1	
	.0001	.051	.012	.063	.058	92.1	
						100.8	$\pm 3.154$

 TABLE V.  
 Uniformity of Vit. B<sub>12</sub> Assay Values Obtained from Various Substances with *L. leichmannii*.

Substance	No. of assays	Vit. B <sub>12</sub> content	Range	Standard error of the mean	Coefficient of variation, %
Vit. B <sub>12</sub> conc.*	14	8.06 $\gamma$ /ml	7.69–8.54	$\pm 0.067$	3.10
USP injectable liver extr. (15 unit)	10	0.590 $\gamma$ /unit	0.545–0.629	$\pm 0.009$	4.64
Wilson's liver L	7	0.389 $\gamma$ /g	0.374–0.416	$\pm 0.006$	3.62

\* Prepared as described previously.

a growth response in *L. leichmannii* on a casein hydrolysate medium,<sup>1</sup> was also found to promote a growth response on the amino acid medium. Twenty-four thousand times more thymidine than vitamin B<sub>12</sub> was required to produce the same growth response. Due to this wide ratio, it is highly improbable that interference from thymidine will be encountered in the assay of most materials for vitamin B<sub>12</sub> activity by the procedure described in this report.

The ability of the method to produce repeatable assay results was determined by running several assays on successive days on the same sample of unknown against crystalline vitamin B<sub>12</sub> solution. The results are presented in Table V. Excellent replicability was found to exist between repeated daily assays. Values from a crude liver fraction were just as consistent as those from a vitamin B<sub>12</sub> concentrate.

*Assay of Various Materials.* Feedstuffs currently employed in experimental chick diets used to study vitamin B<sub>12</sub> at this laboratory were chosen for the development of extraction procedures. The vitamin B<sub>12</sub> content of several of these materials is presented in Table VI.

USP injectable liver extracts were found to need no treatment other than dilution. A range of 87.1 to 2170 m $\gamma$  of vitamin B<sub>12</sub> per USP unit was found from the assay of 10 different products. The results confirm the report by Rickes, *et al.*<sup>13</sup> that a considerable variation exists in the amount of vitamin B<sub>12</sub> to which 1 USP unit is equivalent. In the liberation of vitamin B<sub>12</sub> from animal products 1 g sample is placed in a 125 ml Erlenmeyer flask together with 50 ml of phosphate buffer (pH 6.8), 20 mg of pancreatin suspended in phosphate buffer, and 1 ml of toluene. The materials are well mixed and incubated for 20 hours at 37°C. After incubation the pH is adjusted to 5.5 with 2 N HCl followed by 5 minutes of steaming. The sample is cooled, adjusted to 100 ml and filtered through Whatman fluted filter paper No. 12. With vegetable materials a 2 g sample is placed in a 250 ml Erlenmeyer flask together with 100 ml of acetate buffer (pH 4.5). The mixture is autoclaved for 30 minutes at 20 lb. After cooling, a suspension containing 40 mg of papain and 40 mg of

<sup>13</sup> Rickes, E. L., Brink, N. G., Kominszy, P. R., Wood, T. R., and Folkers, K., *Science*, 1948, **107**, 396.

# Proceedings

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### SECTION MEETINGS

MINNESOTA	
University of Minnesota	November 16, 1949
MISSOURI	
St. Louis University	November 23, 1949
OHIO VALLEY	
Louisville, Kentucky	November 12, 1949
PACIFIC COAST	
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University of Utah Medical School	November 5, 1949
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Tulane University	October 28, 1949
SOUTHERN CALIFORNIA	
California Institute of Technology	November 3, 1949
SOUTHWESTERN	
University of Oklahoma Medical School	November 11, 1949

### Effect of Vitamin-E Deficiency on Amount of Gonadotrophin in the Anterior Pituitary of Rats.\* (17486)

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Associated with a deficiency of vitamin E in males there are degenerative changes in the germinal epithelium and disappearance of spermatogenesis without apparent morphological alteration of the interstitial cells. The failure of vitamin-E deficient female rats to become pregnant is apparently due to a disturbance of the implantation process rather than the absence of ovulation; there is no direct

proof of ovarian dysfunction.(1,2) Since normal function of the ovaries and testes is dependent upon the anterior pituitary, several authors have compared the morphology or the gonadotrophic potency of the anterior pituitary of normal rats and of rats on a diet deficient in vitamin E. In males, van Wagenen(3) found castration changes in the basophils of

\* Aided by grants from E. R. Squibb and Sons, the United States Public Health Service, and the Williams-Waterman Fund of the Research Corporation.

1. Kaunitz, H., and Slanetz, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1947, v66, 334.

2. Blandau, R. J., Kaunitz, H., and Slanetz, C. A., *J. Nutr.*, 1949, v38, 97.

3. Van Wagenen, G., *Anat. Rec.*, 1925, v29, 298.



TABLE II.  
Gonadotrophic Potency of the Anterior Pituitary of Normal and Vitamin-E Deficient Rats.

				Donor rats	Wt of ant. pit.	
Group	Sex	Avg age, days	No.	Body wt,* g	Fresh,* mg/100 g	Dry, mg/100 g
Normal	M	325	12	375 ± 13.4	2.29 ± 0.11	.41
E-deficient	M	315	13	255 ± 12.3	2.86 ± 0.24	.54
Normal	F	388	12	254 ± 8.7	5.11 ± 0.04	.79
E-deficient	F	469	12	237 ± 11.5	4.35 ± 0.20	.71

Hypophysectomized male recipient rats†											
Ant. pit. powder			No.	Source	Dose, mg	Wt of testes,* mg	P‡	Wt of sem. ves.,* mg	P‡	Wt of ant. prost.,* mg	P‡
23						121.3 ± 3.40		4.68 ± .33		10.91 ± .51	
27	Normal ♂	.1	154.2 ± 3.39	<.01	5.90 ± .36	>.6	17.07 ± .71	.1			
29	E-defic. ♂	.1	170.1 ± 1.28		6.14 ± .34		19.05 ± .90				
29	Normal ♀	.2	147.2 ± 3.71	<.02	4.99 ± .23	>.5	16.88 ± .76	>.2			
29	E-defic. ♀	.2	162.6 ± 5.10		5.19 ± .25		17.92 ± .73				

\* Mean and standard error of the mean.

† Average wt range of experimental groups: 40.2 to 41.0 g; average wt of controls: 35.9 g.

‡ Comparison of means calculated according to "t" method of Fisher.

tency, a fine suspension of the dried powder in physiological saline was made so that each 2 cc contained the total amount of the gland to be injected into one rat. The total dose for groups of rats for each day was placed in separate vials which were kept frozen until injected. One-half cc of the suspension was given subcutaneously on each of the 4 consecutive days, starting one day after operation, to a male weanling rat hypophysectomized at 21 days of age by the parapharyngeal approach.<sup>(10)</sup> The rats were sacrificed 24 hours after the last injection; the testes, seminal vesicles and anterior prostates were carefully removed, freed of connective tissue and weighed on a sensitive torsion balance. Any rats with remnants of the pituitary as detected by examination of the sella with a binocular microscope were excluded. A considerable number of hypophysectomized rats was sacrificed prior to the experiments to determine a suitable dose of pituitary powder necessary to initiate hypertrophy of the sex glands. It was found to be 100 µg of the male and 200 µg of the female dried pituitary powder.

**Results.** It will be seen from Table II

that the weight of the testes of the immature hypophysectomized rats which received injections of anterior pituitary glands from either male or female vitamin-E deficient rats was greater than that of rats receiving corresponding doses of the gland from normal male or female rats. The weights of the seminal vesicles and anterior prostates of the rats which received anterior pituitary from normal or vitamin-E deficient male and female donors showed, however, no significant difference.

**Discussion.** It is evident from the foregoing results that there was a significant increase in the gonadotrophic potency of the anterior pituitary glands of vitamin-E deficient rats of both sexes. These findings, however, did not indicate whether the increased gonadotrophin content of the anterior pituitary of the vitamin-E deficient rats was owing to an increased production or to a decreased secretion or to both.

The fact that only the weights of the testes and not those of the anterior prostates or seminal vesicles of the hypophysectomized rats were greater in rats which received injections of anterior pituitary from vitamin E-deficient

the gland. Her results were extended and confirmed by Koneff.(4) The anterior pituitary of female E-deficient rats likewise showed the morphological changes characteristic of spaying, according to Joel.(5) Both Koneff and Joel also described a hypertrophy and a decrease in the number of oxyphils. In conformity with these findings, Nelson(6) reported that the increase in the gonadotrophic potency of the pituitary of E-deficient males approached that of the gland of castrates. He detected no difference in the amount of gonadotrophin in the pituitaries of normal and E-deficient females. However, McQueen-Williams(7) concluded that glands from E-deficient female rats contain about twice as much gonadotrophin as those from normal rats or E-deficient rats receiving the vitamin for 2 weeks or longer. In contrast with the foregoing reports, Biddulph and Meyer(8) stated that pituitary powder from vitamin-E deficient male or female rats produced the same weight change in the ovaries of hypophysectomized rats used for assay as normal pituitary powder from a corresponding sex. However, they believed that male pituitaries from E-deficient rats contained an increased amount of luteinizing (interstitial-cell stimulating) hormone. The gonadotrophin in all these experiments was assayed in immature female rats whereas Rowlands and Singer(9) employed a different method: the production of ovulation in estrous rabbits. According to their findings, the pituitary of the E-deficient female rat contained less gonadotrophin than the normal although this may have depended upon a reduction of a gonadotrophin not detectable in the assays in rats.

With the exception of the experiments of Biddulph and Meyer, all the assays in the work cited above were performed in animals

TABLE I.  
Composition of Vit. E Deficient Diet.

Basal mixture	%
Casein, crude	30
Cerelose	54
Lard, commercial	10
Salt mixture (Hawk-Oser)	4
Celluluration	2
Supplements of basal mixture	mg/kilo
Thiamine chloride	2
Riboflavin	4
Pyridoxine	4
Calcium pantothenate	10
p-Amino benzoic acid	300
Choline	1000
Inositol	1000
Vit. K	4
Oleum percomorphum	200

with intact pituitaries. It seemed of interest to reinvestigate the problem by performing the assays in hypophysectomized male rats so that (a) any contribution of the anterior pituitary of the assay animals to the gonadotrophic effect of the glands of donors could be excluded, and, (b), interstitial-cell stimulation could be better evaluated from effects on the anterior prostate. The female donor groups were of special interest since studies(2) of their ovarian function had already been completed.

*Methods.* Albino rats of a highly inbred stock were maintained on the vitamin-E deficient diet given in Table I from birth until sacrificed. The control rats received the same diet supplemented by 3 mg of synthetic dl-alpha-tocopherol acetate<sup>†</sup> per 100 g of the diet, permitting a daily intake of 300  $\mu$ g in contrast to 30 micrograms of the vitamin afforded by the deficient diet. At average ages ranging from 315 to 469 days, corresponding groups of vitamin-E deficient and control rats were sacrificed, and the anterior pituitary gland from each rat was removed, carefully dissected and weighed. The pituitaries from each group of rats were triturated together in a mortar into a homogenous fine suspension in distilled water, transferred to an ampoule, quickly frozen and dried in a frozen state under high vacuum.

For the bioassay of its gonadotrophic po-

<sup>†</sup> Synthetic dl-alpha-tocopherol acetate was kindly supplied by Dr. Leo Pirk of Hoffmann-La Roche.

4. Koneff, A. A., *Anat. Rec.*, 1939, v74, 383.
5. Joel, C. A., *Monatschr. Geburtsh. Gynak.*, 1943, v116, 288.
6. Nelson, W. O., *Anat. Rec.*, 1931, v56, 241.
7. McQueen-Williams, M., *Anat. Rec.*, 1934, v58 (Suppl.), 77.
8. Biddulph, C., and Meyer, R. K., *Amer. J. Physiol.*, 1941, v132, 259.
9. Rowlands, I. W., and Singer, E., *J. Physiol.*, 1936, v86, 323.

presence of sulphate radicals. Wislocki, *et al.*(2) have shown, however, that hyaluronic acid, a mucopolysaccharide which contains no sulphate radical, also stains with toluidine blue. This gradual change in staining characteristics toward toluidine blue probably indicates a change in the nature of the ground substance.

Micro sections of the rabbit callus from the first to the sixteenth day after fracture were stained with the Hale technic. Hale(3) feels that this stain is specific for acid mucopolysaccharides. An acid polysaccharide such as hyaluronic acid combines with dialyzed iron in an acid solution but a neutral polysaccharide or a protein does not. The bound iron can be demonstrated as Prussian Blue by treatment with potassium ferricyanide and hydrochloric acid. If the slides are counterstained with a red dye such as paracarmine, the acid polysaccharides appear blue against the red background of other tissue structures. The positive reaction for acid polysaccharides parallels those obtained with toluidine blue. Recently injured tissue had little affinity for the dialyzed iron, but by 7 days the formed granulation tissue stained a deep blue strongly suggesting the presence of a high concentration of acid polysaccharides. Staining was most intense at 7 to 10 days, declined thereafter, and was almost disappearing by the tenth to fourteenth day.

Qualitative studies of the 7 day rabbit callus showed a high concentration of sulphate and phosphate ions. A test for the presence of amino sugars with Ehrlich's reagent was strongly positive. Believing that possibly we were staining one of the known acid mucopolysaccharides we attempted chemical isolation. A chemical analysis for hyaluronic acid by viscosimetric methods gave a negative result. Since great loss is entailed in these methods, we incubated tissue sections of the 7 to 10 day rabbit callus with increasing concentrations of hyaluronidase for varying periods of time. The tissue's staining did not change. Viscosimetric analysis by means of

an Ostwald viscosimeter showed no depolymerization of the ground rabbit callus by hyaluronidase.

Following Meyer's procedure(4) attempts were made to isolate chondroitin sulphuric acid from the cartilaginous 7 day rabbit callus. The results were negative although this mucopolysaccharide might logically be present since it is seen so abundantly in mature cartilage. Repeated attempts at identification for chondroitin sulfuric acid gave negative results. A possible explanation may be that procedures for the isolation of mucopolysaccharides entail great loss and as yet, we have not had available, the necessary large quantities of tissue.

In the course of analysis for chondroitin sulfuric acid a granular substance precipitated out of slightly alkaline alcohol. This substance prolonged the Lee White clotting time of blood almost fifteen times. The addition of both protamine and toluidine blue would inhibit this action. An analysis for heparin following Howell's(5) original method yielded a small quantity of protein contaminated crystals which possessed mild anticoagulant powers.

The 7 day rabbit callus was also incubated with lysozyme. Histochemically and viscosimetrically no depolymerization of the ground substance took place. These studies indicate the probable absence of hyaluronic acid and the substrate of lysozyme. The enzyme lysozyme is present in the granulation tissue of the rabbit callus, however, in a concentration of about 84 units after the fifth day after fracture.(6) The presence of chondroitin sulfuric acid is still unsettled, but much larger quantities of tissue must be studied.

We have studied the healing cycle in the rabbit and have sought to identify by histochemical and chemical methods an acid mucopolysaccharide which appears as an intermediary metabolite in the transformation of the interfibrillar ground substance. The studies are incomplete but contribute to the clarification.

4. Meyer, Karl, and Smyth, Elizabeth M., *J. Biol. Chem.*, 1937, v119, 507.

5. Howell, W. H., *Am. J. Phys. Proc.*, 1922-1923, v63, 434.

6. Prudden, J. F., Lane, N., and Meyer, Karl, in press.

2. Wislocki, G., Bunting, G. H., and Dempsey, E. W., *Am. J. Anat.*, 1947, v81, 1.

3. Hale, C. W., *Nature*, London, 1946, v157, 802.



rats might be attributed to an increase in the pituitary follicular stimulating hormone (FSH) without concomitant increase in the interstitial-cell stimulating hormone (ICSH). Such an interpretation would be in conformity with the well known fact that vitamin-E deficiency causes degenerative changes of the germinal epithelium without evident histological or functional changes in the interstitial cells in the testes. It is possible that differences in the quantities of the two gonadotrophins could be revealed if several different dose levels were to be used for the comparisons. However, no conclusion can be drawn from the experiments reported in Table II.

That the anterior pituitary of the vitamin-E deficient female rats also contained an increased amount of gonadotrophic hormone is of interest because of the controversy concerning ovarian function in vitamin-E deficiency. Although the ovaries of old, E-deficient rats showed a greater accumulation of connective tissue and pigmentation than those of controls, observations of ovarian function, such as the production of ova followed by fertilization, revealed no abnormalities. It remains to be investigated, however, whether the increased

gonadotrophin content of the pituitary gland indicates a disturbance of ovarian function or whether a different explanation must be sought.

*Summary.* The gonadotrophin content of the anterior pituitary taken from vitamin-E deficient rats, 315 to 469 days of age, was determined. The weights of testes, seminal vesicles and anterior prostates of groups of male rats hypophysectomized at 21 days of age and subsequently injected with suspensions of frozen-dried anterior pituitaries were compared. The testes of the hypophysectomized rats which received the pituitary suspension from vitamin-E deficient male or female rats were significantly heavier than those of rats which received pituitary powder from the controls. There was no difference in the weights of the seminal vesicles or anterior prostates.

It is concluded that the gonadotrophin content of the anterior pituitary gland is elevated in older vitamin-E deficient male and female rats. The relationship of these findings to the changes in the gonads in vitamin-E deficiency are discussed.

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### A Probable Acid Mucopolysaccharide Present in Granulation Tissue.\* (17487)

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(Introduced by M. Heidelberger)

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This paper is a preliminary report of observations made on the healing of experimental fractures in the rabbit. Certain histological and chemical reactions of the reparative tissue have led us to the belief that there is present in the early granulation tissue of bone repair, an acid mucopolysaccharide which may play a role in tissue healing. Tissue sections of rabbit callus from one to 10 days

after fracture were stained metachromatically with a 1% solution of toluidine blue. Early specimens take up only a small quantity of the dye. As the granulation tissue forms, however, an increasing metachromasia appears, and as the fibrous connective tissue becomes more abundant and differentiated, the ability to stain metachromatically with toluidine blue diminishes. Lison(1) attributed this ability to stain metachromatically to the

\* This work was made possible by a grant through the Office of Naval Research, United States Navy Department.

1. Lison, L., *Soc. de Biol. Compt. Rend.*, 1935, v118.

TABLE I.  
Dietary Regimen.

Ration No.	Type of restriction	Ration fed per mouse per day
1	Caloric	1 g FPSV + 0.5 g sucrose
2	"	1 g FPSV + 1.5 g sucrose
3	Total	1 g optimum ration
4	"	2 g optimum ration
5	"	3 g optimum ration
6	None	Optimum ration <i>ad lib.</i>

that 0.5 g of the mixture contained the following: thiamine 0.3 mg, riboflavin 0.3 mg, pyridoxine 0.3 mg, niacin 1.5 mg, calcium pantothenate 2.0 mg, i-inositol 100 mg, sodium p-amino-benzoate 100 mg, biotin 10  $\gamma$ , folic acid 25  $\gamma$ . In the rations designed to determine the effect of caloric restriction, fat, protein, salts, and vitamins (referred to as FPSV) were fed in the same proportions as they occur in the optimum diet, and the indicated amounts of sucrose were added. Each gram of the FPSV mixture contained casein 0.65 g, salts IV 0.14 g, vitamins 0.029 g and corn oil 0.18 g, enough to reasonably insure the consumption of as much of each per mouse as would be consumed by mice receiving the optimum diet *ad lib.* This being the case, the only constituent deficient in rations 1 and 2 was sucrose. The dietary regimen of each group is listed in Table I. In previous experiments of a similar nature it was learned that mice would die if placed abruptly on the restricted rations, therefore a plan of progressive restriction as outlined in Table II was followed during the first ten days of the experiment. For caloric restriction the FPSV mixture and sucrose were measured separately and blended manually in each ration cup. Groups 1 through 4 received the same quantity of FPSV. For total restriction, decreasing amounts of the optimum diet were fed until the determined level of food consumption was reached. Water was always available. Five days after the start of the experiment, the individual weights of the majority of the animals had reached a maximum (except the optimum *ad lib.* group) and by the seventh day, they had

begun to decline.

Virus: 16 days after beginning the experiment all the test groups were inoculated intracerebrally with 0.03 ml of a saline suspension (Series 98,  $10^{-2}$ ; Series 115,  $10^{-3}$ ) of Theiler's(9) GDVII virus infected brain and cord. All animals were observed daily and weighed twice weekly.

*Results and discussion.* The results are summarized in Table III. Uninoculated mice remained alive throughout the experiment and were alert and active in contrast to those receiving the virus. In the latter group, hind-leg weakness, tremors, ataxia, humped back, and occasionally tonic convulsions, with extension of the hindlegs and flexion of the forelimbs, were observed. During the convulsions, the mice became rigid and cyanotic; death sometimes occurred during these seizures, but more often the mouse recovered. Similar convulsions have been seen in other nutritional deficiencies such as that of tryptophan. In the present experiments, the animals that were paralyzed and those that showed only signs of encephalitis were grouped together in calculating the percentage virus infection.

In Series 98, in which practically 100% of the mice in the control inoculated groups developed signs of the virus infection, Groups 2 and 6 showed this typical infection in only 50% and 43%, respectively, but showed total fatalities in 100% and 95% of the animals. This would seem to indicate that either a diet with low caloric value or a restriction of the optimum diet to 1 g per day definitely alters the signs of infection. But, in these same groups, animals dying without showing any signs of infection survived 5 and 7.5 days (average survival time. AST) respectively while the average incubation period (AIP) of mice which did show the typical picture was 9 and 8.8 days respectively. We assume that in most cases the mice which died without signs of infection actually succumbed to virus invasion. Several lines of evidence support this assumption. Gard(10) has presented evi-

<sup>8</sup> Mazola, fortified with oleum percomorphum (Mead Johnson & Co., Evansville, Ind.) to supply ca. 1800 I.U. vitamin A and 260 I.U. vitamin D per 100 g ration.

9. Theiler, M., and Gard, S., *J. Exp. Med.*, 1940, v72, 49.

10. Gard, S., *J. Exp. Med.*, 1940, v72, 69.

tion of the changes during healing in the organic matrix. To integrate these observations with present knowledge, a working hypothesis must take into account enzyme action, hydrogen ion concentration, and the concentrations of ions such as calcium, carbonate, and phosphate as well as other electrolytes participating in the transformation of the organic matrix. Further studies are indicated and are in progress. Because of the

difficulties involved in obtaining large quantities of healing callus, the identification of the acid mucopolysaccharide is incomplete.

*Summary.* Chemical and histochemical observations concentrate on a mucopolysaccharide which appears to be important in bone healing.

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### Effect of Dietary Restriction on Susceptibility of Mice to Infection with Theiler's GDVII Virus.\* (17488)

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That nutritional deficiencies are frequently accompanied by anorexia is well known. Numerous reports(1-7) have been published which concern the relation of nutritional inadequacies both to bacterial and to viral infections, but the evidence as to whether or not the course of experimental infections is influenced by caloric restriction, or by restriction of an adequate diet, inanition, seems equivocal. Different infective agents, different host species, genetic status of host and of pathogen, different routes of infection, and many other important variables frequently make direct

comparison impossible and analysis of conflicting results exceedingly difficult. The experiments here reported, query further the relative effects of reduced food intake resulting from loss of appetite and of a simple decrease in caloric intake on the course of Theiler's GDVII encephalomyelitis in mice.

*Materials and methods.* Mice: Webster Swiss mice, from our own colony, were used in all experiments. When 3 to 4 weeks old, they were removed from the colony, weighed and distributed in individual screen-bottom cages. Each group contained approximately equal numbers of male and female mice of similar weight and litter mates in any one group were kept to a minimum.

*Diets:* the diet referred to as "optimum" has the following percentage composition; casein 18 $\frac{1}{2}$  sucrose 72.2, salts IV 4,(8) vitamins 0.5, $\frac{1}{2}$  choline chloride 0.3 and corn oil 5. $\frac{1}{2}$  The vitamins were made up with sucrose so

\* Aided by a grant from the National Foundation for Infantile Paralysis.

1. Aycock, W. L., and Lutman, G. E., *Am. J. Med. Sci.*, 1944, v208, 389.

2. Clark, P. F., McClung, L. S., Pinkerton, H., Price, W. H., and Schneider, H. A., *Bact. Rev.*, 1949, v13, 99.

3. Rasmussen, A. F., Jr., Waisman, H. A., Elvehjem, C. A., and Clark, P. F., *J. Inf. Dis.*, 1944, v74, 41.

4. Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *J. Exp. Med.*, 1944, v79, 221.

5. Foster, C., Jones, J. H., Henle, W., and Dorfman, F., *J. Exp. Med.*, 1944, v80, 257.

6. Weaver, H. M., *Am. J. Dis. Child.*, 1945, v69, 26.

7. Metcalf, J., Darling, D., Wilson, D., Lapi, A., and Stare, F. J., *J. Lab. Clin. Med.*, 1949, v34, 335.

† A hot alcohol-extracted, vitamin test casein (General Biochemicals, Inc., Chagrin Falls, Ohio).

‡ We are indebted to Merck & Co., Rahway, N.J., for the crystalline vitamins.

8. Liehstein, H. C., McCall, K. B., Kearney, E. B., Elvehjem, C. A., and Clark, P. F., *Proc. Soc. Exp. Biol. and Med.*, 1946, v62, 279. In these studies the salt mixture was modified by using ZnSO $_4$  insted of Zn Cl $_2$ .

For example, in this series 98, although groups 2 and 6 showed only 50 and 43% respectively of manifest virus infection, the AIP, the AST, and the total fatalities present a picture of almost 100% actual virus infection. The results obtained with Groups 9 and 10 are essentially alike with almost 100% manifest virus infection. A number of mice receiving 3 g of optimum diet per day did not consume this amount from one feeding to the next; it is inferred therefore that their appetites were satisfied, approximating the *ad lib.* group in food consumption. The groups receiving 1 g or 2 g of optimum diet per day devoured their ration completely. The remaining groups (4, 8) with less severe restrictions, showed no significant differences in incidence of paralysis.

*Series 115.* Thirty-five % of the mice in group 6 (1 g optimum diet) died without any signs of the disease. However, the AST (9.0 days) of these mice was less than the AIP (10.2 days) for this group and both the AST and AIP were less than the AIP (11.0 days) of the animals on the optimum *ad lib.* diet (Group 10). Among the remaining groups (2, 4, 8) in this series, typical infection levels ranged from 86% to 95%.

We have therefore almost identical pictures in the two series. In each, the groups receiving restricted amounts of food, whether low in calories, or low in amounts of an optimum diet, showed no significant decrease in infection as we have defined the term. The exact figures for the two series are not directly comparable since in series 98, the inoculum was a  $10^{-2}$  suspension whereas in series 115, this was a  $10^{-3}$  suspension. This difference resulted in a longer AIP and a slightly longer AST in the latter series. Our results are

not precisely in accord with the reports of others who have found that restriction of the caloric value or reduction of food intake produces results similar to but less marked than those obtained with thiamine deficient diets. We would emphasize that our criteria of infection do not rest solely on visible signs and that the other authors used different viruses, either Theiler's FA or the Lansing mouse adapted poliomyelitis virus. Only with the Lansing strain did they obtain definitive results.

Although Theiler (11) has reported that the more obvious physical and chemical properties of mouse encephalomyelitis virus and human poliomyelitis are "identical", immunologically, and on the basis of species susceptibility and the pathological lesions produced, the viruses are definitely different. The dissimilarity in behavior of GDVII and Lansing poliomyelitis virus in mice when the animals are under various nutritional stresses appears to be another manifestation of differences between these viruses. Our experience indicates that the course of infection with the Lansing strain is more sensitive to nutritional stress than is infection with Theiler's GDVII virus.

*Summary.* Although diets restricted either calorically or by total food intake do influence the course of infection in mice inoculated with Theiler's GDVII virus, in that frank signs of infection, *i.e.* paralysis, encephalitis, are frequently not evident, yet the total fatalities, the average incubation period, and the average survival time are not modified appreciably by these deficiencies.

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11. Theiler, M., *Med.*, 1941, v20, 443.

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TABLE II.  
Plan of Restriction.

Group No.	Days after start of experiment					
	0	3	4	5	7	10
1 and 2	Opt. <i>ad lib.</i>	Opt. 3 g	FPSV 1 g + sucrose 2 g	FPSV 1 g + sucrose 1.5 g	FPSV 1 g + sucrose 1 g	FPSV 1 g + sucrose 0.5 g
3 and 4	"	"	"	"	FPSV 1 g + sucrose 1.5 g	FPSV 1 g + sucrose 1.5 g
5 and 6	"	"	Opt. 2.5 g	Opt. 2 g	Opt. 1.5 g	Opt. 1 g
7 and 8	"	"	"	"	Opt. 2 g	Opt. 2 g
9	"	"	Opt. 3 g	Opt. 3 g	Opt. 3 g	Opt. 3 g
10	"	Opt. <i>ad lib.</i>	Opt. <i>ad lib.</i>	Opt. <i>ad lib.</i>	Opt. <i>ad lib.</i>	Opt. <i>ad lib.</i>

TABLE III.  
Summary of Fatalities and Virus Infection.

Series No.	Group No.*	Ration No.	No. of mice	Fatalities, † %	Virus infection, %	Total fatalities, %	AST, ‡ days	AIP, § days
98	1 U	1	7	0	—	0	—	—
	2 I	1	20	50	50	100	5.0	9.0
	3 U	2	7	0	—	0	—	—
	4 I	2	21	0	100	100	—	7.7
	5 U	3	7	0	—	0	—	—
	6 I	3	21	52	43	95	7.5	8.8
	7 U	4	7	0	—	0	—	—
	8 I	4	20	0	100	100	—	7.9
	9 I	5	14	7	93	100	12.0	8.9
	10 I	6	14	0	100	100	—	8.9
138								
115	1 U	1	7	0	—	0	—	—
	2 I	1	21	14	86	100	10.0	9.5
	3 U	2	7	0	—	0	—	—
	4 I	2	21	5	95	100	7.0	9.1
	5 U	3	6	0	—	0	—	—
	6 I	3	20	35	65	100	9.0	10.2
	7 U	4	7	0	—	0	—	—
	8 I	4	20	5	95	100	10.0	9.9
	9 I	5	14	7	93	100	7.0	10.0
	10 I	6	14	14	86	100	8.0	11.0
137								

\* U = uninoculated; I = inoculated.

† Without signs of infection.

‡ Average survival time of mice dying without signs of GDVII infection.

§ Average incubation period of mice developing signs of GDVII infection.

|| 1 mouse only.

dence that the AIP is an index of virus titer. On numerous occasions we have assayed the brains and cords of infected, undernourished mice at intervals after inoculation and when moribund but without typical signs of infection. This central nervous system material when subsequently inoculated into normal mice has shown virus activity indicating that the virus does proliferate in the CNS of deficient mice even though the typical signs of

infection are not seen. Further, in some experiments, deficient inoculated mice which have survived beyond the normal incubation period without visible evidence of infection have succumbed to the disease with the typical picture when fed optimum rations. As criteria of infection, therefore, we are inclined to weigh not only paralysis and visible signs of encephalitis, but also the AIP, the AST and total fatalities.

by GDVII antiserum.<sup>†</sup>

*Experiments with the MEF Virus.* The strain was obtained in 1945 in its 8th mouse passage through the courtesy of Dr. P. K. Olitsky, Rockefeller Institute, New York City. The virus was carried in mice in this laboratory using the same technic as previously described for the Lansing virus, except that passages were made rapidly with freshly harvested material and no intervening storage. On 4 occasions the virus was examined for pathogenicity in rhesus monkeys by intracerebral injection of 1 cc of 10% viral mouse brain-cord suspension. One monkey received the 10th, one monkey the 15th, one monkey the 19th, and one monkey the 20th serial mouse passage. The first monkey injected with the 10th serial mouse passage became paralyzed on the 14th day but no active virus could be recovered from the cord of the paralyzed animal by transfer to another rhesus monkey or to a group of mice. The other three monkeys remained free from any symptoms of disease. Two of the symptomless monkeys were tested, two months later, for immunity by intracerebral challenge with Ayrcock virus (0.5 cc 1:100). Both developed typical paralysis. The third monkey was used for the production of hyperimmune anti-MEF serum.

As previously described for the Lansing virus, the MEF virus at all times maintained its typical clinical, biological and serological characteristics.<sup>‡</sup>

*Experiments with the Y-SK Virus.* The

<sup>†</sup> This statement refers to the strain under investigation in this paper and not to any rhesus-nonpathogenic, high-titred, serologically atypical possible variants which may or may not have been derived from the original Lansing virus.(4,5,6)

4. Jungeblut, C. W., *Proceedings Fourth International Congress for Microbiology*, Copenhagen, 1947, p. 259.

5. Enright, J. B., and Schultz, E. W., *Proc. Soc. Exp. Biol. and Med.*, 1947, 66, 541.

6. Sanders, F. K., *Proceedings Federation of American Societies for Experimental Biology*, Atlantic City, 1948, p. 309.

<sup>‡</sup> Reference is made to the original MEF strain and not to a rhesus-nonpathogenic, high-titred possible variant which has previously been described.(4)

virus was obtained in 1947 through the courtesy of Dr. G. Dalldorf. It was propagated in this laboratory by rapid serial passage in mice as previously described for the MEF virus. Intracerebral tests for pathogenicity in rhesus monkeys were carried out with various generations, inoculating two monkeys with the 5th and one monkey each with the 10th and 15th serial mouse passages. All monkeys developed typical flaccid paralysis within 9 to 13 days after injection, except one monkey inoculated with the 5th passage and another monkey inoculated with the 10th passage. No attempts were made to carry the strain further from monkey to monkey but all efforts to recover the virus by inoculation of mice with the cord of paralyzed monkeys uniformly failed.

The Y-SK virus was obtained once more in 1948 through the courtesy of Dr. J. Melnick, Yale University, New Haven, Conn., the material being received in form of frozen pooled mouse brains and cords. The original material proved highly infectious for rhesus monkeys, since 7 of 8 monkeys injected intracerebrally on various occasions between 1948 and 1949 developed typical paralysis. The strain was carried in mice in this laboratory by rapid transfer from mouse to mouse using freshly harvested brains and cords. Tests for rhesus pathogenicity were made with the 6th, 11th and 15th serial mouse passage. One of 2 monkeys each injected with the 6th or 11th mouse passage developed paralysis, as did 2 of 3 monkeys injected with the 15th mouse passage. Again, virus in mouse-pathogenic form could not be recovered from the cords of these animals when completely paralyzed.

During the time of these observations the Y-SK virus maintained its typical clinical, biological and serological characteristics, which made it indistinguishable from the Lansing and MEF virus, except for its somewhat lower virulence in mice.

*Conclusions.* The data presented above demonstrate that strains of mouse-adapted human poliomyelitis virus may exhibit wide variations in the degree of their pathogenicity for rhesus monkeys. The Lansing virus, for instance, harvested from remote mouse pas-

# Monkey Pathogenicity of Various Strains of Murine Poliomyelitis Virus.

## I. Experiments with Lansing, MEF, and Yale-SK Group.\* (17489)

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Adaptation of human poliomyelitis virus from monkey or man to rodents results in the production of murine strains which differ significantly in the degree of their pathogenicity for mice and rhesus monkeys. One group, with low potency in mice, *i.e.* Lansing, MEF and Yale-SK virus, retain to a large extent their original ability to paralyze rhesus monkeys, even though continuous passage from mouse to mouse may cause some deterioration of this property. (1) Another group, Columbia-SK and MM virus, with high potency in mice, produce in rhesus monkeys rarely flaccid paralysis but set up a subclinical infection characterized by massive intraneural and extraneural virus multiplication as well as by abundant antibody formation. (2) A third group, transferable only to suckling mice, apparently has no pathogenicity whatsoever for rhesus monkeys. (3) The following communication deals with a study of the pathogenicity for rhesus monkeys of serial mouse passages of viral strains belonging to the first group, *i.e.* Lansing, MEF and Y-SK virus.

*Experiments with the Lansing Virus.* The strain was obtained in 1945 in its 36th mouse passage through the courtesy of Dr. G. Dall-dorf, State Laboratory, Albany, N. Y. The virus has since been carried in this laboratory in mice by making transfers at irregular intervals from glycerinated material stored in the ice-box. A 10% suspension of infected brain and cord tissues was used routinely and 0.03 cc injected intracerebrally into 11-14 g Swiss albino mice. During the past 3 years the virus was examined for its pathogenicity for rhesus monkeys on 3 occasions. In each

case a 10% suspension was prepared from freshly harvested brain and cord of a paralyzed mouse and 1 cc of the supernatant, after light centrifugation, was injected intracerebrally into rhesus monkeys weighing about 2500-3000 g. One monkey was injected with the 41st, 2 monkeys with the 57th, and one monkey with the 63rd serial mouse passage. None of the injected monkeys showed any signs of disease, such as fever, weakness or paralysis. Since the animals were subsequently used for the production of hyperimmune anti-Lansing serum there was no opportunity for examining any sections of the spinal cord for possible subclinical lesions or for testing these animals for immunity by reinfection with potent simian virus.

In subsequent experiments the same strain was used in attempts to infect 2 cynomolgus monkeys by intracerebral injection with 1 cc of a 10% mouse brain suspension from the 74th serial mouse passage. Both monkeys remained free from symptoms. It will be of considerable interest to further examine this apparently monkey-nonpathogenic Lansing strain for its ability to induce protection, in the absence of paralysis, against a pathogenic strain.

At the time these observations were made the virus always presented the typical clinical, biological and serological characteristics of the strain. Paralysis occurred only after intracerebral and not intraperitoneal injection, infected mice showed flaccid paralysis of the front or hind legs, the incubation period varied from 48 hours to 21 days, and the potency of the virus fluctuated between  $10^{-1}$  and  $10^{-2}$  with a mortality rate of between 80 and 90%. Moreover, the virus was neutralized by hyperimmune Lansing antiserum (obtained from Dr. Isabel Morgan, Johns Hopkins University, Baltimore) and by a majority of normal and polio-convalescent sera collected in the metropolitan area, but not by Col-SK antiserum or

\* Aided by a grant from the Sister Elizabeth Kenny Foundation.

1. Theiler, M., *Medicine*, 1941, v20, 43.
2. Jungeblut, C. W., and Sanders, M., *J. Exp. Med.*, 1940, v72, 407; *ibid.*, 1942, v75, 611.
3. Dalldorf, G., Sickles, G. M., Plager, H., and Gifford, R., *J. Exp. Med.*, 1949, v89, 567.



FIG. 1. Col-SK virus (guinea pig) first monkey generation cynomolgus No. 7 lumbar cord.  
 FIG. 2. MM virus (mouse) first monkey generation cynomolgus No. 12 lumbar cord.  
 FIG. 3. High Lansing virus third monkey generation cynomolgus No. 32 cervical cord.  
 FIG. 4. EMC virus third monkey generation cynomolgus No. 30 lumbar cord.

rus.(4) For purpose of control, 2 highly virulent Theiler strains of spontaneous mouse encephalomyelitis, *i.e.* GDVII and FA virus, were also examined for possible pathogenic effects in cynomolgus monkeys. The EMC virus was obtained from Dr. J. Warren, Army Medical School, Washington, D. C., the Schultz High Lansing virus from Dr. Edwin Schultz, Stanford University, California, and the 2 Theiler strains from Dr. Max Theiler, Rockefeller Foundation, New York.

**Methods.** Ten per cent viral brain suspensions were prepared from freshly harvested current mouse passages of the above mentioned strains and 1 cc of the supernatant, after light centrifugation, was injected intracerebrally into young cynomolgus monkeys weighing between 1000 and 2000 g. In the case of Col-SK virus, in addition to mouse virus preparations, viral brain suspensions were also used

from paralyzed cotton rats or guinea pigs. The injected cynomolgus monkeys were kept under close observation and symptoms noted. Animals showing paralysis were sacrificed at the height of their symptoms and the cord was removed for histological study as well as for virus recovery. Freshly prepared monkey cord suspensions were further passed to new cynomolgus or rhesus monkeys (1 cc 1:10 intracerebrally) and, at the same time, were titrated out in mice by both the intracerebral and intraperitoneal route. Serial monkey passages were maintained over 2, 3 or 4 generations depending upon the absence or presence of paralytic symptoms. The results obtained are brought together in Diagram I and in Table I and are further illustrated in a series of microphotograms (Fig. 1-4).

**Col-SK Virus.** It will be noted that Col-SK virus, whether harvested from paralyzed cot-



sages, gave no evidence of being pathogenic for rhesus monkeys in repeated tests. The MEF virus, originally rhesus-pathogenic, apparently suffered a progressive loss of its pathogenicity for rhesus monkeys during serially maintained rapid mouse passages. The Y-SK virus, finally, seemed to have undergone but little change in its pathogenic power for rhesus monkeys as the strain was passed rapidly through mice. It is realized that the described phenomena may not always be duplicated in different laboratories since the Lansing virus has frequently been reported

by others as being fully rhesus-pathogenic. Yet the authenticity of our strains is attested by the fact that all three murine strains had fully preserved their biological and immunological characteristics. The observed differences in the degree of pathogenicity for rhesus monkeys and in the extent to which murine virus returns to mice from paralyzed monkeys may be due to individual differences among strains or reflect an attainment of different levels of perfection in the adaptive process.

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## Monkey Pathogenicity of Various Strains of Murine Poliomyelitis Virus. II. Experiments with Col. SK-MM Group.\* (17490)

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In the preceding paper (1) data have been presented to show that the Lansing, MEF and Y-SK virus, upon serial passage in mice, may undergo various degrees of deterioration in their ability to produce flaccid paralysis in intracerebrally infected rhesus monkeys. This reduction in monkey pathogenicity, however, was not associated with any demonstrable changes in other biological or immunological properties. It became next of interest to investigate whether the Col-SK-MM group of viruses—agents which since their initial transfer to mice have shown little or no paralyzing power in rhesus monkeys—would be capable of inducing paralytic infection, with central nervous system lesions, in cynomolgus (Java) monkeys.

The cynomolgus monkey was chosen for this purpose since the response of this animal to poliomyelitis virus differs in many respects from that observed in rhesus monkeys. Whereas the rhesus monkey, as a rule, is susceptible only to intracerebral infection and the virus

remains strictly localized in the central nervous system, the cynomolgus monkey may be infected by peripheral portals of entry, including the gastrointestinal tract, with the production of a systemic disease characterized by wide extraneural distribution of the virus and by fecal excretion. Moreover, isolation of poliomyelitis virus from fresh human or non-human sources often succeeds better with the cynomolgus than with the rhesus monkey. (2)

The following communication deals with a study of the pathogenicity for cynomolgus monkeys of current mouse passages of Col-SK and MM virus. Included are also experiments with 2 viruses of as yet uncertain identity but with close serological relationship to Col-SK and MM virus. These are the virus of so-called encephalomyocarditis (EMC virus) (3) which was isolated from chimpanzees suffering from unknown myocardial disease, and the Schultz High Lansing strain, presumably a "high" variant of the original Lansing vi-

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3. Schmidt, E. C. H., *Am. J. Pathol.*, 1948, v24, 97.

4. Enright, J. B., and Schultz, E. W., *Proc. Soc. Exp. Biol. and Med.*, 1947, v66, 541.

\* Aided by a grant from the Sister Elizabeth Kenny Foundation.

1. Jungeblut, C. W., *Proc. Soc. Exp. Biol. and Med.*, 1949, in press.

ton rats, mice or guinea pigs, proved to be highly pathogenic for cynomolgus monkeys in paralyzing 5 of 6 such monkeys following intracerebral injection; on the other hand, paralysis occurred in only one of 2 injected rhesus monkeys. When carried from the first to the second monkey generation, the number of "takes" was greatly diminished, only one of 5 injected cynomolgus monkeys and 3 of 7 injected rhesus monkeys developing paralysis. In the third monkey generation there was a further reduction of monkey pathogenicity in that paralysis occurred in one rhesus monkey only within a group of 5 rhesus and 2 cynomolgus monkeys. No paralysis was observed in the fourth monkey generation consisting of 2 rhesus monkeys.

The lesions observed in the spinal cord of paralyzed cynomolgus or rhesus monkeys are described in Table I. In 8 out of 12 cases they were those of severe acute poliomyelitis, consisting of widespread ganglion cell necrosis in the anterior horn, with numerous examples of neuronophagia, extensive inflammatory reactions around the blood vessels and occasional areas of hemorrhage. In 3 cases the pathology was limited to more sharply defined localizations of ganglion cell necrosis in the anterior horn or central grey with poorly developed neuronophagia and but little inflammatory response. One case, finally, showed a mild poliomyelitic process with evidence of beginning repair characterized by extensive gliosis. A constant feature of the severely damaged cords was the close proximity between perivascular infiltration and ganglion cell necrosis. Pathological examination of the brain of 2 cynomolgus monkeys (not listed in Table I), which had developed paralysis following intracerebral injection with 1 cc of a  $10^{-2}$  and  $10^{-3}$  dilution of Col SK mouse virus, showed occasional lesions in the brain stem and a few scattered collections of cells in the motor cortex, near the tract of inoculation. A typical picture has been selected for illustration in microphotogram 1.

The results of virus titrations in mice injected with cord suspensions from the paralyzed monkeys are given in Table I. In general, the virus obtained from paralyzed monkeys was considerably less mouse-patho-

genic than virus harvested from paralyzed mice but higher titers were usually observed in cynomolgus rather than in rhesus monkeys. With successive monkey passages virus recoveries, especially from rhesus monkeys, were of the order commonly observed with low-titred murine strains belonging to the Lansing, MEF and Y-SK group. However, when virus harvested from the original group of injected mice was further passed to a new set of mice, complete restoration of potency to the titer characteristic of Col-SK mouse passage virus, i.e.  $10^{-9}$  i.c. and  $10^{-8}$  i.p., became at once apparent. The loss of viral potency suffered by monkey passage was therefore merely a transient depression of virulence and the virus had not been transformed into a permanently low strain.

*MM Virus.* Two cynomolgus monkeys injected with the mouse virus became paralyzed within 3-4 days but attempts to carry the virus into the second monkey generation were unsuccessful. One of the paralyzed monkeys presented typical poliomyelitic lesions in the cord (Fig. 2), the other showed only ganglion cell necrosis with scanty inflammatory reaction. The virus recovered from the cords of both paralyzed cynomolgus monkeys proved of exceptionally low virulence in mice but could be restored to its original high level of murine potency in the next mouse passage.

*High Lansing (Schultz) Virus.* The mouse virus paralyzed 2 cynomolgus monkeys each in the first, second and third monkey generation but failed to transmit to the fourth generation. Severe poliomyelitic lesions (Fig. 3) were found in 4 of the 6 paralyzed monkeys, whereas in the remaining 2 animals only occasional areas showed ganglion cell necrosis without inflammatory reaction. The cords of all paralyzed monkeys yielded virus of moderately high virulence for mice.

*EMC Virus.* The mouse virus paralyzed one of 2 cynomolgus monkeys in the first generation, both cynomolgi of the second, and one of 2 in the third generation; no paralysis was obtained in the fourth generation. Typical poliomyelitic lesions (Fig. 4) were found in the cords of 3 animals, the remaining monkeys presenting an atypical picture of occasional chromatolysis of ganglion

TABLE I. Symptoms, Pathology, and Viral Content (Cord) of Monkeys Paralyzed by Col-SK, MM, High Lansing (Schultz) or EMC Virus.  
Virus content of cord titrated in mice

Monkey No.	Virus injected	Monkey generation	Symptoms	Pathology, CNS	i.e.	i.p.
Cynomolgus C2	Col-SK cotton rat No. 5	I	Generalized paralysis, 4 days	Ganglion cell necrosis	10-5	10-5
Rhesus AS48	"	I	Paralysis both legs, 7 days	Died 16 days. Poliomyelitis with repair	Neg. 10-1	Neg. 10-1
Cynomolgus C4	Col-SK mouse No. 422	I	Paralysis left arm and both legs, 3 days	Recovered	N.d.†	N.d.†
" C5	"	I	Paralysis right leg, 3 days	Typical poliomyelitis	10-5	10-5
" C1	Col-SK guinea pig No. 81	I	Paralysis left arm and leg, 6 days	"	10-5	10-5
" C6	"	I	Weakness 5th day, died 6th day	"	10-3	10-1
" C7	"	I	Paralysis right leg, 13 days	"	10-1	Neg. 10-1
Rhesus AS50	Col-SK monkey C5	II	Paralysis right leg, 6 days	"	Neg. 10-1	Neg. 10-1
" AS51	"	II	Paralysis right arm, 13 days	"	10-1	Neg. 10-1
Cynomolgus C21	"	II	Weakness 3rd day died 3rd day	Ganglion cell necrosis	10-5	10-5
" C22	"	II	Generalized paralysis, 4th day	"	10-3	10-1
Rhesus AS60	" (7	II	Paralysis left arm, 7 days	Typical poliomyelitis	10-3	10-1
" AS62	" AS51	III	Paralysis left arm, 8 days	"	10-3	10-1
Cynomolgus C11	MM mouse No. 138	I	Generalized paralysis, 3 days	Ganglion cell necrosis	Neg. 10-1	Neg. 10-1
" C12	"	I	Paralysis both legs, 4 days	Typical poliomyelitis	10-3	10-3
" C19	High Lansing mouse No. 9	I	Generalized paralysis, 3 days	"	10-5	10-5
" C20	High Lansing mouse No. 9	I	Generalized paralysis, 3 days	Ganglion cell necrosis	N.d.†	N.d.†
" C25	High Lansing monkeys C19-C20	II	Paralysis left leg and arm, 4 days	"	10-5	10-5
" C26	High Lansing monkeys C19-C20	II	Paralysis left leg and right arm, 6 days	Typical poliomyelitis	10-5	10-5
" C31	High Lansing monkeys C25-C26	III	Generalized paralysis, 5 days	"	10-5	10-5
" C32	High Lansing monkeys C25-C26	III	Generalized paralysis, 4 days	"	10-5	10-5
" C17	EMC mouse No. 70	I	Paralysis both legs, 3 days	"	10-5	10-5
" C18	"	I	Paralysis left leg and arm, 4 days	Died 24 days. Not examined	N.d.†	N.d.†
" C23	EMC monkey C17	II	Generalized paralysis, 5 days	Typical poliomyelitis	10-5	10-5
" C24	"	II	Paralysis left arm and leg, 5 days	Atypical	10-5	10-5
" C30	EMC monkeys C23-C24	III	Paralysis left arm and both legs, 4 days	Typical poliomyelitis	10-5	10-5

† N.d. = not done.

groups of virus must not be overlooked. For, whereas murine Lansing, MEF and Y-SK virus can often, if not always, be maintained in an unbroken series of monkey generations, murine Col-SK, MM, High Lansing and EMC virus produce paralysis in monkeys only for a limited number of passages. The abrupt extinction of paralyzing power for primates, despite the presence of a potentially virulent agent and the manifestation of extensive lesions in the central nervous system of the infected monkey, constitutes an as yet unsolved problem. The problem is further accentuated by the fact that monkey passage fails to bring about a permanent conversion of the "viscerotropic" Col-SK strain into the "neurotropic" Y-SK strain. If one contrasts the obvious depression of virulence in monkeys—a frequent phenomenon for the Col-SK-MM group of virus—with the ease with which the same viruses can be propagated in mice, one is confronted with several possible conclusions. Either different murine strains of human poliomyelitis virus possess different innate degrees of pathogenicity for monkeys and mice; or, some of these viruses have suffered an irreversible loss of their intrinsic monkey pathogenicity, subsequent to their experimental adaptation to rodents. A third consideration would be that in the case of the Col-SK-MM-EMC-Mengo group of viruses we are deal-

ing with a serologically homogenous group of aberrant members of the poliomyelitis family which, though capable of producing paralytic poliomyelitis in monkeys and probably also in man,(6) are genetically not of human but of rodent origin in nature. This hypothesis might receive support from the recent demonstration of the regional occurrence of virucidal antibodies against EMC and MM virus in the sera of wild rats.(7) Obviously, further study of the complex problem is necessary in order to provide the correct answer.

**Conclusions.** 1) Col-SK, MM, High Lansing and EMC virus, harvested from remote rodent passages, produce typical poliomyelitis in intracerebrally infected cynomolgus monkeys. 2) Two strains of highly virulent Theiler virus, GDVII and FA, are non-pathogenic for cynomolgus monkeys. 3) The Col-SK-MM group of virus can be carried in cynomolgus monkeys only for a limited number of passages. 4) Judging from serological and biological characteristics, monkey passage fails to bring about a permanent conversion of Col-SK virus into Y-SK virus.

6. Jungeblut, C. W., in preparation.

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## Artificial Kidney. II. Construction and Operation of an Improved Continuous Dialyzer.\* (17491)

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The insertion of a continuous dialyzer (artificial kidney) in the circulatory system of an animal or a human in uremia has been shown to be capable of removing nitrogenous

waste products(1-10) and to result in the adjustment of electrolyte equilibrium.(1,3,8,

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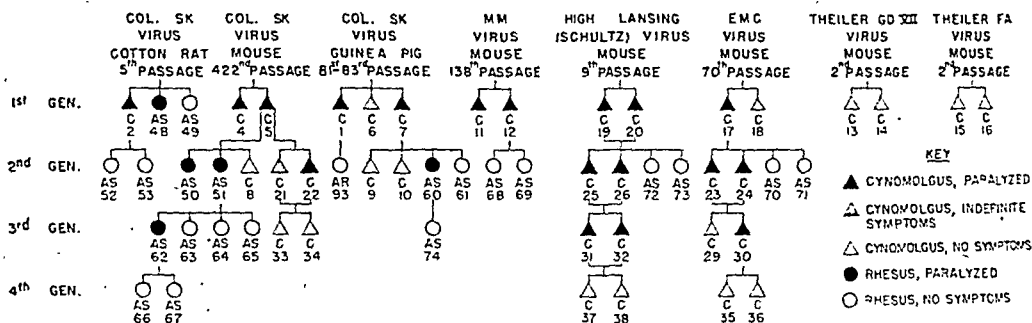
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5. Alwall, N., Norviit, L., and Steins, A. M., *Acta med. Scand.*, 1949, v132, 477.

DIAGRAM I



MONKEY PATHOGENICITY OF THE COL-SK-MM GROUP OF VIRUSES  
FIG. 5.

cells but no perivascular infiltration. No evidence of demyelination could be detected in the cords of the 4 monkeys examined. Virus recovered from the cords of paralyzed monkeys, in all 3 generations, proved consistently moderately pathogenic for mice.

*Theiler Virus (GDVII and FA).* Two cynomolgus monkeys were injected intracerebrally with each of the 2 strains harvested from fresh mouse passages. None of the 4 animals showed any signs of illness during one month observation. Two monkeys were sacrificed at that time and cord sections examined histologically. No lesions of any kind were found in these cords.

*Immunological Tests.* The immunological properties of the various murine strains, following their passage through monkeys, were studied: 1) by examining a number of representative sera from paralyzed or symptomless monkeys for their ability to neutralize Col-SK and Y-SK murine virus in mice, 2) by determining the extent to which Col-SK monkey virus, harvested from infected monkey cords, could be inactivated in mice by known Col-SK and Y-SK antisera, 3) by reinfecting all surviving symptomless monkeys with simian poliomyelitis virus. Suffice it to say that neutralization of Col-SK murine virus was readily obtained by the majority of sera collected from cynomolgus or rhesus monkeys infected with Col-SK, MM, High Lansing or EMC virus (but not with Theiler virus), whereas the same sera failed to neutralize Y-SK murine virus. In harmony with this fact, Col-SK virus, prepared from infected cynomolgus cords, was

fully inactivated by known Col-SK antiserum but not regularly by known Y-SK antiserum. In the reinfection experiment, finally, a group of 8 cynomolgus monkeys surviving asymptomatic infection with either Col-SK virus (C8, C10, C33, C34) High Lansing virus (C37, C38) or EMC virus (C29, C35), together with one normal rhesus control monkey, were challenged by intracerebral injection of simian poliomyelitis virus (1 cc 1:10 Aycock rhesus passage virus). All animals developed complete or partial paralysis within 1-3 weeks.

*Discussion.* The results obtained in this study leave little doubt that the murine strains at present classified as the Col-SK-MM-EMC-Mengo group of virus are highly pathogenic for cynomolgus monkeys and induce in this animal an experimental disease which is symptomatologically and pathologically similar to that produced by the Lansing-Y-SK group of murine virus in rhesus monkeys. African grey monkeys (*Cercopithecus aethiops*) have also been reported as developing paralysis more frequently than rhesus monkeys following infection with Mengo virus.<sup>(5)</sup> Although these facts seem well enough established,<sup>†</sup> significant differences in the degree of such monkey pathogenicity between the two

5. Dick, G. W. A., and Smithburn, K. C. *Br. J. Exp. Path.*, 1948, v29, 547; Dick, G. W. A., *ibid.*, 1948, v29, 550.

<sup>†</sup> The fact that Col-SK virus paralyzes cynomolgus monkeys with the pathological picture of poliomyelitis in the spinal cord has been confirmed by Dr. J. D. Verlinde, Leiden, Holland, who will publish his findings elsewhere.

an opening through which blood may enter the space between the pieces of cellophane and flow in a uniform film to the opposite end where it may leave the apparatus through the second pair of steel plates. Dialyzing solution flows in a countercurrent direction through the grooved spaces in the rubber pads on both sides of the thin layer of blood contained between the two pieces of cellophane. As many such units as desired are easily assembled one above another and clamped with a simple holder composed of 2 flat steel plates. When so assembled all units are automatically connected in parallel through suitable interconnecting holes at both ends of the rubber pads. By appropriate placement of small slips of cellophane between the individual units, they may be connected in series or in series parallel, several combinations being possible. This makes it possible to alter the resistance of the "kidney" to the available flow of blood. For dogs weighing between 15 and 20 kg in which blood is drawn from the femoral artery and returned to the femoral vein the best assembly has been found to be a total of 8 units, 2 parallel sets of 4 units being connected in series.

If the "kidney" is properly assembled, there will be no leakage of blood into the dialyzing solution, or between the rubber pads. To detect assembly errors, air is introduced into the spaces to be occupied by the blood at 150-200 mm of mercury above atmospheric pressure. If no decrease in air pressure occurs, the apparatus is considered satisfactory. No holes have yet been found in the cellophane sheets and leaks have never developed during the dialysis. DuPont No. 300 non-moisture-proof cellophane sheets may be purchased cut to size.

The connections between "kidney" and animal are shown in Fig. 2. A rubber tube (B) sufficiently long to lead to the arterial cannula is connected to the blood spaces at one end of the "kidney." A similar tube (H) leads from the other end to the venous cannula. In this line is placed a simple glass air trap (G). It is also convenient to place glass T connections (C and F) in both arterial and venous lines. These are used to fill the "kidney" with blood and to administer intra-

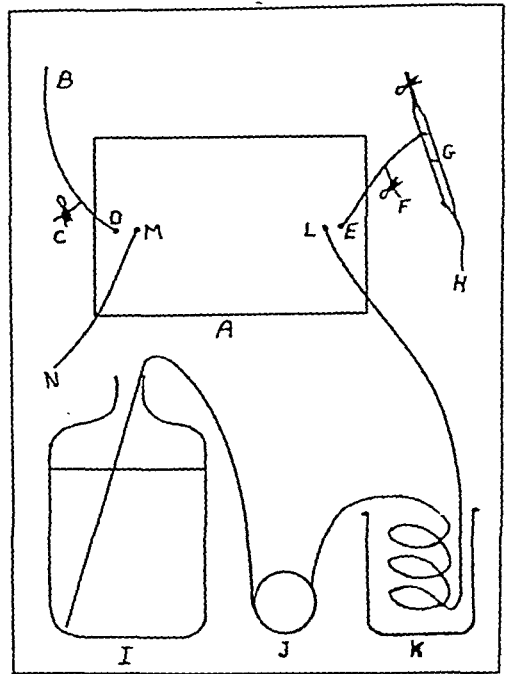


FIG. 2.

Diagram of connections of the artificial kidney to the animal and to the source of dialyzing solution. See text for explanation of letters.

venous solutions. During sterilization these 2 T tubes may be connected to the dialyzing solution inlet (L) and outlet (M). Steam at 5-15 lb. pressure is run through the arterial tube (B), the "kidney," glass trap (G), and the venous lines (H) for 30 minutes. Following sterilization the "kidney" is cooled and washed by running cold water for several hours through the dialyzing solution spaces only. Just before use the assembled "kidney" is rinsed by passing 6-10 liters of sterile saline through the blood spaces. We have found it convenient to place the kidney on an ordinary hospital bedside table and to reserve the lower part of the table for an apparatus designed to deliver dialyzing solution warmed to 37.5°C at any desired rate. This apparatus consists of a small centrifugal pump<sup>‡</sup> (J) controlled by a rheostat which propels dialyzing solution from a 5 gallon bottle (I) through a 20 foot length of 1/4 inch diameter copper tube coiled in a thermostatically controlled

<sup>‡</sup> Eastern Engineering Co., New Haven, Conn., Model B-1.

10,11). This continuous dialysis of blood has proved valuable(12,13) in the isolation of substances which are present in blood in low concentrations. The method may also be applied to fundamental physiological studies. (14,15) For such dialysis to be reasonably successful the apparatus used should contain a large area of dialyzing membrane so that the rate of dialysis may be rapid and the process be completed in 1-4 hours. The volume of blood which it contains should be relatively small and constant in order to avoid undue changes in the subject's blood volume. The dialyzing solution on the other side of the membrane should be in a closed system. This is necessary in order to keep a certain amount of  $\text{CO}_2$  in solution which makes possible a physiological pH and avoids precipitation of calcium phosphate. The apparatus must be easy to clean, assemble and sterilize and should preferably be inexpensive.

Several artificial kidneys have been described.(1,2,7,12,14,16) Although differing in constructional details they are all characterized by the use of long lengths of cellophane tubing (sometimes greater than 100 feet) and the design most widely used at the present(1) is of an undesirably large size. In a previous communication(17) we described a new design of a continuous dialyzer

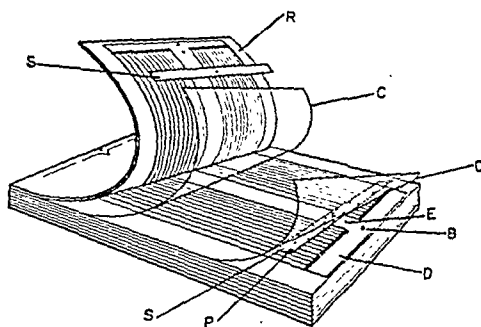


FIG. 1.

Diagram of the artificial kidney. The top rubber pad of an assembly of 4 units has been bent back to show the relative position of the inner parts.

R—Grooved rubber pads.

C—Cellophane sheets.

S—Stainless steel plates between the two sheets of cellophane. These plates are separated one millimeter by projections (P) in one of the plates.

D—Depression in rubber pads to accommodate the steel plates.

B—Interconnecting tubes for blood. The holes in the steel plates fit exactly over B.

E—Interconnecting tubes for dialyzing solution.

which met the above requirements more closely than any previously described. Although it has been used in many successful experiments it had one main disadvantage in that the blood being dialyzed was exposed to a large surface of rubber which accelerated clotting and necessitated coating the rubber with a non-wetting silicone resin. This involved a considerable amount of work. The present report describes an improved model in which the above objection is overcome and in which the dialyzing efficiency has been greatly increased.

The dialyzer is composed of a variable number of units, each unit consisting of two identical longitudinally-grooved rubber pads<sup>1</sup> (12 inches by 18 inches by 3/16 inch), between which are placed 2 sheets of cellophane (Fig. 1). The two pieces of cellophane are separated at either end by 2 identical pairs of stainless steel plates which at one end provide

17. Skeggs, L. T., Jr., and Leonards, J. R., *Science*, 1948, v108, 212.

† The authors wish to acknowledge the invaluable assistance of the Honek Machine Co., Barberton, Ohio, for machining the specially designed steel mold and of A. E. Sidnell and the personnel of the Seiberling Latex Products Co., of Akron, Ohio, for molding the rubber pads.

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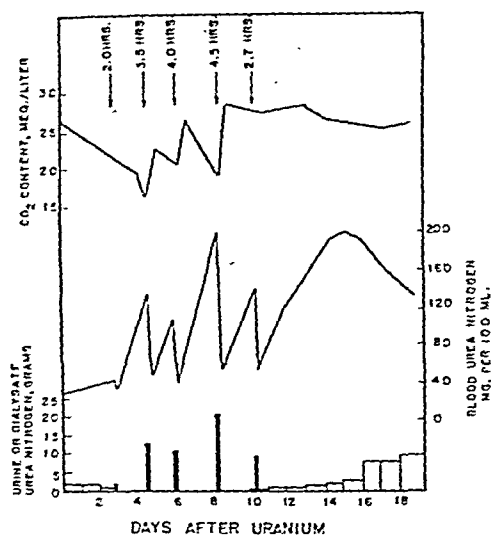


FIG. 3.

The effect of treatment with the artificial kidney on the blood urea nitrogen and the  $\text{CO}_2$  content of plasma of a dog given a dose of uranium nitrate (5 mg per kg) which is usually lethal. The arrows represent dialyses of the durations indicated. Note the restoration of the blood urea nitrogen to almost normal levels and the elevation of the  $\text{CO}_2$  content towards normal. The solid black bars at the bottom of the chart represent the urea nitrogen removed from the animal by the artificial kidney. This animal recovered.

thal.(20,21) The data on one of these animals are presented in Fig. 3. Three days after

the injection of the uranium, the animal became anuric. At this time treatment was instituted and a total of 5 dialyses at 1-2 day intervals were successfully accomplished. The blood urea nitrogen was restored to nearly normal levels by each dialysis. A total of 57 g of urea nitrogen were recovered in the dialyzing solution. The ability of the artificial kidney to regulate acid-base balance is illustrated by the fact that the  $\text{CO}_2$  content of the plasma which was decreased as a result of the inadequate renal function was repeatedly elevated toward normal levels. Chloride, sodium, and potassium remained within normal limits. Following the fifth dialysis, 10 days after the dose of uranium, the dog began to excrete urine and survived without further treatment.

**Summary.** An improved type of continuous dialyzer specifically designed for use as an artificial kidney has been described. A thin film of blood is made to flow between 2 sheets of cellophane supported between rubber pads which are grooved to allow passage of a dialyzing solution. Details of its operation are given and its application in the treatment of acute renal insufficiency in dogs is described.

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### Intravenous Infusions of Concentrated Combined Fat Emulsions into Human Subjects.\* (17492)

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The successful development of a method for the preparation of a 10% combined fat emulsion and the results after clinical investigation of this emulsion as reported by us warranted further studies in the preparation and administration of more concentrated fat emulsions.(1) The advantages to be derived from the more concentrated intravenous fat preparations, increased caloric potential in

reduced volume, would constitute a further advance in the field of parenteral fat nutrition. The present report is devoted to the method of preparation of two combined fat emulsions with fat in 15 and 20% concentrations and a study of the effect of their intravenous infusion on human subjects.

#### *Preparation of Concentrated Fat Emulsions.*

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\* Aided by a grant from the Ben Lewis Fund in Experimental Surgery.



TABLE I.  
Composition of Dialyzing Solution for the Dog.

Milliequivalents per l			
Na <sup>+</sup>	148	Cl <sup>-</sup>	126
K <sup>+</sup>	3	HCO <sub>3</sub> <sup>=</sup>	24
Ca <sup>++</sup>	5	HPO <sub>4</sub> <sup>-</sup>	2
Mg <sup>++</sup>	3	Lactate <sup>-</sup>	7
Total	159	Total	159
Glucose: 100 mg/100 ml			
CO <sub>2</sub> gas to pH 7.35-7.45			
Preparation of Concentrated Stock Solutions			
Sol. A		Sol. B	
G per l		G per l	
KCl	9.0	NaHCO <sub>3</sub>	84.0
CaCl <sub>2</sub>	11.1	Na <sub>2</sub> HPO <sub>4</sub>	5.7
MgCl <sub>2</sub> · 6H <sub>2</sub> O	12.2	NaOH	9.6
NaCl	269.0		
Glucose	40.0		
Lactic acid	27.1 ml (assay 85-90%)		

500 ml each of solutions A and B are added to 19 liters of distilled water for use. The exact amount of lactic acid required to bring the pH of the diluted dialyzing solution to pH 7.3-7.5 is determined for each lot.

water bath (K), and then to the "kidney" at inlet tube (L). After passing through the "kidney," the dialyzing solution may be collected via tube (N).

The dialyzing solution is prepared to contain the electrolyte composition of the extracellular fluid of the normal dog with the addition of 100 mg of glucose per 100 ml. This composition is calculated from actual analyses of dog plasma obtained in this laboratory over the past 10 years and reported in part by Muntwyler *et al.* (18,19) Solution A (Table I) contains a small excess of lactic acid which upon mixing with the bicarbonate of solution B yields the required amount of dissolved CO<sub>2</sub> to maintain the pH at 7.35-7.45. This makes possible the presence of calcium, phosphate and bicarbonate in physiological concentrations without precipitation.

Twenty dogs have been dialyzed using the above apparatus. Morphine (0.5 mg/kg) or Merperidine Hydrochloride (Demerol) (50-150 mg) was injected subcutaneously and the femoral artery and vein exposed under local anesthesia (0.5% Pontocaine Hydrochloride). A plastic catheter 4 mm in diameter and 4-6

inches long is inserted through the femoral vein into the vena cava. Three mg of heparin per kg of body weight was injected intravenously through this catheter and the femoral artery cannulated. The "kidney" was then filled with heparinized donor blood, connected to the arterial and venous cannulae and blood allowed to flow through the dialyzer. At the end of the dialysis 15 ml of 1% protamine sulfate is slowly injected intravenously. This is usually adequate to restore the blood clotting time to a value of less than 15 minutes. In some animals an additional 50-100 mg of protamine sulfate were found necessary. The cannulae may then be removed. It is imperative that the clotting time be carefully watched if serious hemorrhages are to be avoided.

An arterial pressure of 100 mm Hg resulted in a blood flow of 150-250 ml per minute. Dialyzing solution flows at 400 cc per minute and is under a negligible pressure. The area of cellophane is 12,000 cm<sup>2</sup> which together with a total of 400 cc of blood in the "kidney" gives an average thickness of the film of blood of 0.66 mm. Under these conditions the clearance of urea from the dog's blood is usually about 50 ml of blood per minute (when calculated in a manner exactly analogous to the usual clinical method) and in occasional experiments has been as high as 60-80 ml per minute. It is of interest that creatinine dialyzes through cellophane at a rate only half as great as urea.

Most of the animals tolerated the dialysis without observable effects. Hemolysis did not occur. However, in a few cases the blood pressure decreased to as low as 30-50 mm but returned to normal within 1 to 3 hours. The cause of this effect has not been determined and is now under investigation.

In order to demonstrate the effects of treatment with the artificial kidney, dogs were given 5 mg per kg of anhydrous uranyl nitrate subcutaneously, a dose which is usually le-

¶ Kindly supplied by Paul Lewis Laboratories, Milwaukee, Wis.

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21. Gustafson, G. E., Koletsky, S., and Free, A. H., *Arch. Int. Med.*, 1944, v74, 416.

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19. Mellors, R. C., Muntwyler, E., and Mautz, F. R., *J. Biol. Chem.*, 1942, v144, 773.

TABLE II.  
Immediate Effects from Infusions of Concentrated Fat Emulsions into Human Subjects.

Emulsion	Volumes infused (ml)	g fat infused	No. of patients infused	No. of infusions given	Reactions.					
					<1°	Temperature rise 1°-1.9°	2°-2.9°	>3°	Chill	Vomiting
15%	500 to 600	77.5 to 93.0	14	23	8	3	5	7	7	2
	700 to 900	108.5 to 139.5	2	9	3	0	3	3	2	0
	1000	155.0	2	7	1	1	2	3	1	0
20%	500 to 600	101.0 to 121.2	7	12	4	4	3	1	4	1
Totals			25	51	16	8	13	14	14	3

elevation above the initial pre-infusion temperature but were not corrected for changes due to the disease of the patient. The incidence of chills totalled 14 of all infusions given. Of the latter the occurrence of 7 cases of chills were correlative with high temperature and the administration of the emulsion at the rate of 80 drops per minute. Upon reduction of the rate of infusion these same subjects on other occasions did not repeat the chill when re-infused with the fat emulsion. Chills were the severest type of reaction encountered in the present series. Upon the administration of 50 mg of either pyribenzamine or benadryl the latter reaction was considerably reduced. Vomiting, headache and dizziness occurred in 4 instances and were in association with a chill. Other reactions such as coughs, allergies and fatty taste did not occur in this series. There were also no fatalities. (Table II).

The values for hemoglobin, hematocrit cell volume and red and white cells tended to fall below their initial values during the infusion phase of the emulsion and returned to their original levels shortly before or after the termination of the infusion. The erythrocyte sedimentation rate showed an increase in rapidity of sedimentation which average 25% to 35% greater than the pre-infusion sedimentation time. It also returned to its initial level. The differential white count was not indicative of any trend. The specific gravities of blood and plasma showed a temporary

dilution effect due to the infusions followed by a return to normal limits (Table III).

Changes of blood pressure in response to the injection were variable. During the infusion period there was generally a slight elevation of blood pressure followed by a moderate fall which persisted for 2 hours after completion of the infusion. The elevation of systolic pressure did not exceed 20 mm Hg and the fall in systolic pressure ranged from 10 mm to 25 mm Hg. The diastolic pressure tended to fall towards the end of the infusion but also returned to its normal level. There were no falls in blood pressure which indicated any tendencies toward the development of shock.

Counts of chylomicrons were made from peripheral venous blood under dark field illumination with a net micrometer in the eyepiece of the microscope. In the post absorptive state the pre-infusion count varied from zero to 10 chylomicrons. After the infusion was started there was a rapid rise in the number of chylomicrons to a peak. At about the mid-point of the infusion the chylomicrons began to decrease in number and return slowly to the initial level.

Consecutive urine studies were made in 25 infusion tests. In no case was a positive test for blood or urobilinogen obtained which was attributable to the fat emulsion. Chemical tests for fat in the urine were negative. Dark field examination of the urine for chyl-

TABLE I.  
Composition of 15% and 20% Concentrated Combined Fat Emulsions.

Conc. fat emulsion	% composition				G fat per liter	Calories per liter
	Coconut oil	Gelatin	Glucose	Amigen		
15%	15.5	2.20	5.4	3.6	155.0	1720
20%	20.2	2.66	5.5	2.2	202.0	2100

The successful preparation of the 15% and 20% combined fat emulsions was accomplished by adequate and thorough homogenization and emulsification procedures as described in our previous report on the 10% combined fat emulsion, whereby the homogenizer apparatus adapted to medical usage and the technic of manufacture of sterile emulsions were presented in detail. (1)

The constituents of the emulsions are solutions of 10% protein hydrolysate, 50% glucose, 6% intravenous gelatin (Knox-P-20) and refined coconut oils. All except the latter are available as sterile non-pyrogenic solutions in sealed flasks. The refined coconut oil is autoclaved before mixture with the other emulsion ingredients. In the present studies 15% and 20% concentrations of fatty oil were obtained in the preparation of the combined fat emulsions resulting in caloric values of approximately 1720 and 2100 calories respectively per liter of emulsion. The percentage composition of the various ingredients which make up the emulsions are listed in Table I with fat as the prime contributing factor to the increased caloric value of each preparation.

Intravenous gelatin was used exclusively as the emulsifying agent requiring between 2 and 3% for thorough emulsification of the 15% and 20% concentrations of fatty oil. The stability of both combined fat emulsions has been observed for a period of more than 7 months without "creaming" or "oiling out". The particle size of the emulsions was within the one micron range and active Brownian movement was exhibited on microscopic examination.

*Method of Study.* The combined fat emulsions were administered without selectivity to hospital patients who were being treated for a variety of surgical conditions. Several of these patients ran a chronic low grade fever.

The studies were started in the morning with the subject in the postabsorptive state after a fasting period of 14 hours. Before the infusion was started a sample of blood was taken, the morning urine was obtained, and temperature, pulse, respirations and blood pressure were noted. The infusion was then given intravenously at a specific rate ranging from 20 to 80 drops per minute and varying in different patients. In every case the amount infused was 500 ml of either emulsion. In several subjects a second infusion was also given in the late afternoon. During the course of the infusion and throughout the post-infusion phase until the next morning, blood and urine samples were taken and the above mentioned clinical notations were made at regular intervals. The following tests were made routinely on the blood; hemoglobin, red cell count, white cell count with differential, sedimentation time, chylomicron count and specific gravity. Clinically, subjective and objective constitutional reactions relative to the infusion were studied. X-rays of the lungs were taken in series for any lesions due to fatty infiltration, pulmonary irritation or pulmonary emboli. Final check examinations were made 2 weeks after completion of the infusions for hemolytic anemia and for possible liver or kidney damage. Surgical liver and lung biopsies were obtained from several subjects who received the emulsion. These specimens were prepared for microscopic analysis by staining with osmic acid, Sudan IV and hematoxylin-eosin.

*Results.* The 15% and 20% combined fat emulsions were administered intravenously into 25 human subjects for a total of 51 infusion studies. Details relative to the method of administration, volume infused, temperature response and constitutional reactions are supplied in Table II. Temperature changes were computed according to the degree of

microns showed a moderate elevation in the number of fat particles. In 4 infusion studies with the 15% fat emulsion and in 3 with the 20% fat emulsion, positive reactions were obtained for acetone varying from 1+ to 2+ in 2 or 3 of the intermediate urine specimens examined during the course of the infusion. During the post-infusion phase the tests for acetone occasionally showed a faint trace and remained negative thereafter. There were no positive tests for diacetic acid. There were no indications by these tests of any greater acetone spillage occurring in recipients of the 20% fat emulsion as compared with recipients of the 15% fat emulsion.

Follow-up studies for hematologic complications in subjects who received either single or multiple infusions showed no signs of hemolytic anemia. Likewise x-rays of the lungs gave no evidence of fatty emboli or pulmonary irritation. Liver and lung biopsy specimens failed to show any organic lesions which could be attributed to the intravenous infusion of fat. Liver and kidney function studies showed no pathologic alterations which were attributable to the emulsions.

*Comment.* The effects of the intravenous infusion of either the 15% or 20% combined

fat emulsions were in many respects similar to those noted in our clinical studies on the infusion of the 10% combined fat emulsion.(1) These pertained particularly to hematologic changes, blood pressure response and follow-up observations on liver, lung and kidney function. Constitutional reactions such as high temperatures or chills averaged 27% as compared with 9% with the more dilute fat emulsion. About 50% of the reactions encountered in this series were found to be due to the speed with which the emulsion was administered, the fast infusions being associated with the chill reaction. The temperature elevations could be accounted for on the basis of a sudden plethora of fat. In any case the concentrated fat emulsions required cautious careful administration.

*Summary.* Two types of combined fat emulsions, one containing 15% fat and the other 20% fat, were infused into 25 human subjects. A total of 51 of these concentrated fat emulsions were administered intravenously. The incidence of constitutional reactions was 27%. A variety of laboratory and clinical observations were made in conjunction with these infusions.

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### Malignant Lymphoid Tumors in Orchidectomized Mice Receiving Hypophyseal and Ovarian Grafts at Various Ages.\* (17493)

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While studying the effects of anterior hypophyseal and ovarian transplants on mammary growth in male castrate mice of strain A, we noted the occurrence of lymphoid leukemia and lymphosarcoma in a number of animals bearing these grafts.(1) The present report deals with the incidence of these tumors in the various experimental groups.

\* The investigation was supported by a research grant from the National Cancer Institute, United States Public Health Service.

1. Silberberg, R., and Silberberg, M., *Proc. Soc. Exp. Biol. and Med.*, 1949, v70, 510.

*Material and methods.* The experiments have been described in detail in a previous paper.(1) Briefly, 161 male mice of the closely inbred strain A kept on a standard diet of Purina Laboratory Chow and water were castrated at the age of 3 weeks. The animals were divided into two age groups: *I. Younger age group* grafted at the age of one month: Twenty-eight castrates received four anterior hypophyses each; 26 received 2 or 4 ovaries each; 25 received a combination of 4 hypophyses and ovaries. *II. Older age group* grafted at the age of 7 months: Twenty-eight

TABLE III.  
Effects of Intravenous Infusions of Combined Fat Emulsions into 2 Subjects. A—Received 15% combined fat emulsion. B—Received 20% Combined fat emulsion.

Time (min.)	Amount infused	Subject	Temp.	Pulse	Blood pressure	Hemoglobin	R.B.C. count (million)	W.B.C. count (thousand)	Hematocrit	Sedimentation time	Chylomicron count	Urine acetone
0	0	A	98.4	60	130/80	13.5	3.95	5.8	42	26	12	neg.
		B	98.8	74	160/92	14.6	4.75	7.0	46	23	0	neg.
60	100	A	98.4	60	130/80	14.0	4.20	6.2	42	23	150	neg.
		B	99.0	78	168/92	14.8	4.75	6.8	41	23	100	neg.
120	225	A	98.6	68	154/78	15.0	3.80	6.4	40	38	350	1+
		B	99.0	80	184/88	14.6	4.68	5.0	39	34	400	neg.
180	350	A	99.0	76	130/88	14.0	3.70	6.4	38	41	300	1+
		B	100.0	86	186/74	14.0	4.70	5.6	41	34	400	2+
240	500	A	102.4	100	136/84	14.5	3.65	6.6	41	45	260	3+
		B	102.0	104	178/78	13.8	4.90	5.7	45	34	200	1+
360	—	A	100.6	84	130/88	14.5	3.68	5.8	43	27	100	neg.
		B	102.6	106	182/90	14.0	4.10	6.0	46	30	80	1+
480	—	A	100.0	100	156/70	14.5	3.95	5.6	43	30	100	neg.
		B	102.0	94	188/74	14.2	5.00	6.6	40	28	50	neg.
600	—	A	100.4	96	132/88	15.0	3.45	6.0	42	36	20	neg.
		B	100.0	92	160/80	14.4	4.92	6.8	46	30	60	neg.
720	—	A	100.0	88	110/80	14.8	3.90	6.2	40	34	50	neg.
		B	98.4	84	174/88	14.6	4.80	7.4	48	28	0	neg.
24 hr	—	A	98.8	72	116/80	14.8	3.95	5.2	43	30	0	neg.
			98.6		178/96	13.8	5.00	7.8	40	27	8	neg.

TABLE I.  
Incidence of Malignant Lymphoid Tumors and Time of Appearance of the Neoplasms in the Various Experimental Groups.

Type of graft	Age at grafting (mo.)	Age at death of all mice (mo.)			Animals with lymphoma			Animals with preleukemia change		
		No. of mice	Age (mo.) of appearance		No. of cases	%	Mean	No. of cases	%	Mean
			Mean	Range						
I. Hypophysis	1	28	16.4	9-20	7	25.0	16.6	3	10.7	17.3
II. Ovaries	1	26	19.2	9-24	7	26.9	20.0	3	11.5	20.0
III. Hypophysis and ovaries	1	25	17.4	9-22	9	36.0	18.7	2	8.0	19.5
IV. Hypophysis	7	28	19.3	12-25	3	10.7	22.0	3	10.7	20.7
V. Ovaries	7	25	16.8	12-26	2	8.0	15.0	4	16.0	16.5
VI. Hypophysis and ovaries	7	29	15.7	12-24	4	13.8	17.5	3	10.3	17.7
										16-18
										13-24
										18-21
										18-22
										12-23
										14-22

that grafts may remain alive for a considerable length of time. The following photomicrographs 1-4 demonstrate well preserved hypophyseal and ovarian grafts. As had been found in regard to mammary cancers in these animals,(1) the development of malignant lymphoid tumors could not always be correlated to the state of preservation of these transplants at the time of necropsy. It must thus be assumed that grafts may exert an effect for some time and then undergo regression.

The incidences of malignant lymphoma, the mean age at death, and the age range at death in the various experimental groups are given in Table I.

*Younger age group:* Transplants of anterior hypophysis had a leukemogenic effect of about the same order (25%) as ovarian grafts (26.9%). However, ovaries were slower in calling forth this result than were hypophyseal transplants, the mean age at death of animals of the former series being 20 months as compared with 16.6 months in the latter group. Anterior hypophysis grafted together with ovaries produced malignant lymphoma about 40% more often, namely in 36% of the animals, than either hypophysis or ovaries alone, and the mean age at death in this group was 18.7 months.

*Older age group:* 10.7% of the castrates bearing transplants of anterior hypophysis had lymphomas at the mean age of 22 months; 8% of the animals bearing ovarian grafts alone developed lymphoid tumors at the age of 15 months, and neoplasms were observed in 13.8% of the mice bearing grafts of anterior hypophysis together with ovaries. In this group, the mean age at death was 17.5 months.

*Discussion.* In castrate male mice of strain A bearing anterior hypophyseal grafts, malignant lymphoid tumors were found to develop in increased numbers. This leukemogenic effect of anterior hypophysis was apparently not mediated through the ovaries since it was present in castrates which did not carry ovarian grafts. The mode of action of the anterior hypophyseal hormone in this type of leukemogenesis is unknown. Tentatively, however, the following explanation may be

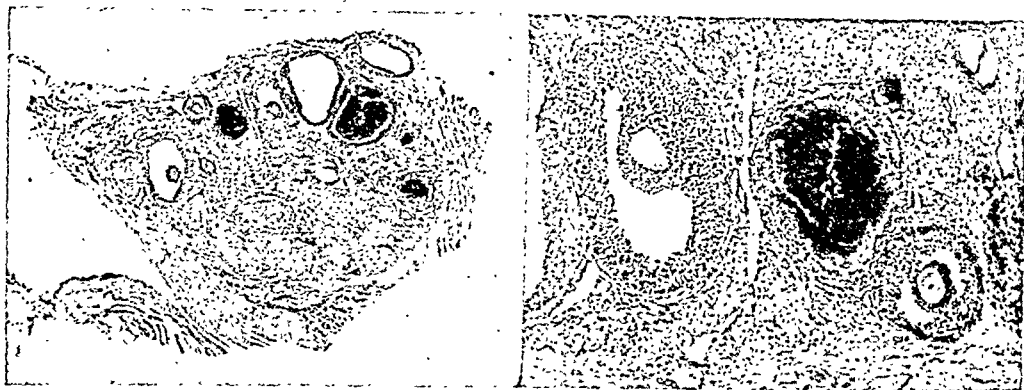


FIG. 1 and 2.

Section through an ovarian graft 21 months after transplantation. Fig. 1 (left) magnification 70X. Fig. 2 (right) magnification 345X.

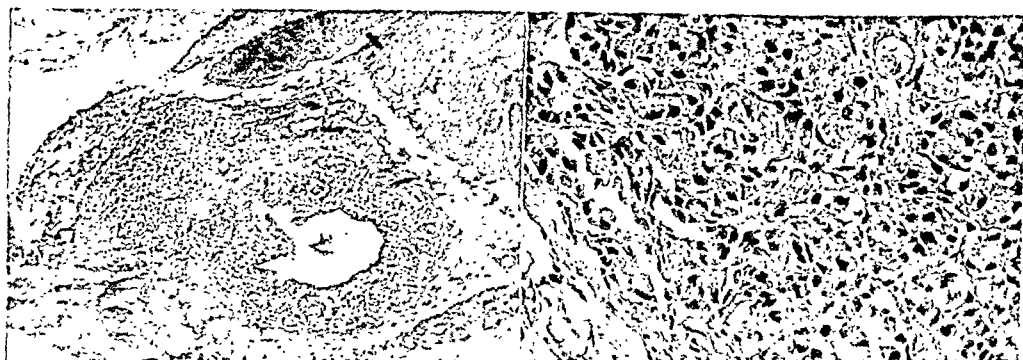


FIG. 3 and 4.

Section through an hypophyseal graft 14 months after transplantation. Fig. 3 (left) magnification 25X. Fig. 4 (right) magnification 93X.

castrates received anterior hypophyses; 25 received ovaries; and 29 received a combination of 4 hypophyses and ovaries. All grafts were obtained from freshly killed animals closely related to the host (syngenesiotransplants).<sup>(2)</sup> The glands were transplanted into a subcutaneous pocket of the chest wall.

**Observations.** In untreated mice of strain A which is known to have a 3.8% incidence<sup>(3)</sup> of spontaneous lymphoma only an occasional case of malignant lymphoid tumor was noted. These tumors with or without leukemia manifested themselves in large neoplasms of the mediastinum or mesentery, or in

diffuse infiltration of lymph nodes, spleen, liver, kidney, lung or heart, leukemic blood picture, or in a combination of several of these findings. Only those cases were classified as malignant lymphoma in which gross and microscopic changes in liver, kidney, lung, heart, and frequently also in blood smears were sufficiently advanced to permit a clear cut diagnosis. Cases in which the microscopic findings were merely suggestive of lymphoma but not definite were listed as preleukemic. Since the present experiments were originally not designed to include observations on leukemia, the bioassay of suspected cases of malignant lymphoid disease, which would have been desirable, was not made. Mice showing merely hemopoiesis were grouped among the negatives. Microscopic examination showed

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3. Kirschbaum, A., and Kaplan, H. S., *Science*, 1944, v100, 360.

## Voluntarily Ingested Sodium Chloride as a Lymphagogue in the Rat.\*† (17494)

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In the course of studies on factors influencing the rate of lymph flow, experiments were carried out to determine the effect of fasting, feeding, and alterations in electrolyte intake on the formation of thoracic duct lymph in the unanaesthetized rat. The results of these experiments indicate that the ingestion of 1% NaCl solution by the fed animal causes an increase in rate of lymph flow which is out of proportion to that seen in control animals.

**Methods and Materials.** Male rats of the Long-Evans strain maintained under comparable environmental conditions were prepared for lymph collection by the method of Bollman, Cain and Grindlay.<sup>(1)</sup> This technique involved the insertion of a plastic cannula into the thoracic duct immediately caudal to the left crus of the diaphragm, at which point the cannula was tied in place, the delivery end being drawn out through the abdominal wall. In these experiments, a ventral midline abdominal operative approach was employed. The rat was then placed in a cage<sup>(2)</sup> which permitted *ad libitum* food and fluid intake, but which restrained activity to the point of allowing collection of lymph samples in the unanaesthetized state. The animals were anaesthetized for the preparation of the fistula by means of intraperitoneal administration of a 2% aqueous solution of sodium pentobarbital in the amount of 7 mg/100 g body weight. Following recovery from the anaesthetized state, the animals were

placed on free oral intake of the regular laboratory diet (XIV) or were fasted as indicated. Each group of animals, as indicated in the tables, was given access to tap water or 1% NaCl for drinking purposes.

**Results.** The experimental results are presented in Tables I and II. Table I presents the results of experiments showing the rate of lymph flow in the fasting and fed animals with free access to water, as compared with the rate of lymph flow in the fasted and fed animal given 1% NaCl for fluid intake, and, finally, the effect of alternation for 24-hour periods of water and 1% NaCl in the fed animal. It will be noted that lymph flow is least in the group of animals fasted and given access to water. Animals fed, with access to water, or fasted with oral intake of 1% NaCl produced comparable amounts of lymph as expressed in ml of lymph per hour per 100 g body weight. On the other hand, rats given access to both food and 1% NaCl for drinking purposes markedly increase their lymph flow to an amount 5-6 times the volume of that of the fed animal on water. The difference in rate of lymph flow is seen most markedly in the last group of animals in this table in which water and 1% NaCl were alternated for 24-hour periods. The output of lymph during the period on saline solution is markedly higher than is the output during water ingestion.

The increase in lymph flow produced by oral intake of 1% NaCl increases with duration of the experiment. Table II presents the average daily lymph volumes in a group of rats maintained on water and 1% NaCl in the fed state. It will be noted that the lymph flow in the animals maintained on water is maximal on the second day and falls off gradually, whereas the flow of lymph in the animals maintained on NaCl increases daily to an amount which exceeds 50% of the body weight of the animal. In one in-

\* Assisted by grants from the Board of Research of the University of California and the National Heart Institute of the United States Public Health Service.

† A portion of the experimental data was presented at the meeting of the American Association of Anatomists at Philadelphia in April, 1949.

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2. Bollman, J. L., *J. Lab. Clin. Med.*, 1948, v33, 1348.



offered: Anterior hypophyseal hormone by stimulating the adrenal cortex may cause a prolonged depletion and atrophy of the lymphoid tissue. This atrophy may become a stimulus to or may represent a step in leukemogenesis,(4-8) since malignant lymphoma is often preceded by atrophy of lymphoid tissue. The leukemogenic effect of the anterior hypophyseal hormone is probably not due to increased output of estrogen by the adrenals, because the mammary glands of these castrates failed to develop cancer or marked acinar proliferation in contrast to conditions found in the castrates carrying ovarian grafts.(1) As to the formation of lymphomas by transplanted ovaries, the results are in agreement with those obtained by the administration of estrogens in mice of various ages,(9) and may be attributed to the production of such hormones by the ovarian grafts. The increased incidence and the shortened latent period of lymphoma in mice bearing hypophyseal grafts together with ovarian transplants seem to represent a cumulative effect of the two hormones given off by the grafts. Whether or not the mechanism by which estrogens produce lymphoid tumors is in any way connected with that initiated by anterior hypophyseal hormone or whether two different mechanisms are involved, is unknown.

In the older age group, the incidence of lymphomas induced by anterior hypophysis fell to 10.7%. Thus the susceptibility to these neoplasms declined somewhat, as the age of the animal at the time of grafting advanced. However, the difference in the mean lymphoma

age in the two age groups (5.4 months) agrees closely with the difference in age at the time when the transplants were made (6 months' difference). The latent periods of tumor formation were thus similar in both series. Of the castrates of the older age group carrying ovarian grafts 8% developed lymphoid tumors, and of those bearing ovaries together with anterior hypophysis 13.8% had these neoplasms. However, these tumor incidences are probably not truly representative of the effects of these two types of grafts in the older age group: There was a decrease in the lymphoma incidence and a lowering of the lymphoma age, whereas one would expect the lower lymphoma incidence to be associated with a prolongation of the latent period. This apparent discrepancy may, however, be explained by the relatively early age at death of the mice in these two groups. The total incidence of malignant lymphoid tumors and the mean lymphoma age would probably have been higher if the animals of these groups had reached an older age. There was no obvious cause for the relatively short life spans of these mice particularly since none of them developed mammary cancer. As to the possible role of an age factor it may be stated: However small the incidence of induced lymphoma in the older age groups, it was still larger than the zero incidence of mammary cancers observed in the same mice.

**Conclusions.** Mice of strain A orchidectomized before weaning and bearing grafts of anterior hypophysis, of ovaries, or of anterior hypophysis together with ovaries from the age of one month on, developed malignant lymphoid tumors. Anterior hypophysis seems to exert its leukemogenic effect (1) through the ovaries and (2) independently of the grafted ovaries, possibly through the adrenal cortex. Transplantation of ovaries together with anterior hypophysis resulted in the highest incidence of malignant lymphomas. The age factor plays in this type of leukemogenesis a less important role than in the production of mammary cancers by anterior hypophyseal and ovarian grafts.

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9. Silberberg, M., and Silberberg, R., a. *Arch. Path.*, 1949, v47, 340; b. *Arch. Path.*, 1949, v48, 557.

rates of thoracic duct lymph flow in unanaesthetized male rats maintained under the experimental conditions of fasting and feeding, with free access to either water or 1% NaCl solution.

2. Thoracic duct lymph flow is at a low level in the fasted animal with free access to water. The rate of flow is increased by feeding or access to 1% NaCl solution. The rate of flow in the *fed* animal with free access to 1% NaCl is increased appreciably over that

of the controls, and out of proportion to any hitherto employed method for *voluntarily* increasing lymph flow in the otherwise normal animal. In some instances, the lymph flow of the NaCl treated animal exceeded the body weight of the animal in a 24-hour period.

3. Alternation of periods of access to water and saline solution produced corresponding low and high rates of lymph flow in the fed animals.

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### Specificity of Paper Partition Chromatography for Analysis of Free Amino Acids in Unhydrolyzed Urine.\* (17495)

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The paper partition chromatographic technique devised by Consden, Gordon, and Martin(1) may be more specific for detecting free amino acids in urine than microbiological methods. The number of amino acids found by microbiological assay in unhydrolyzed urine excreted by normal human subjects is greater than the number detected by paper partition chromatography.

Paper partition chromatography has been used to investigate the amino acids excreted in the urine by normal subjects and selected cancer patients. Aliquots of a 24-hour urine collection or of pooled samples obtained over a 5 or 6 day period were standardized by Kjeldahl analysis to contain either 10 mg of total nitrogen or by the Van Slyke ninhydrin method(13,14) to contain 0.5 mg of  $\alpha$ -amino nitrogen per milliliter. All samples were stored at 3°C under toluene. Aliquots re-

quiring concentration were lyophilized and re-diluted to the desired volume. Other aliquots were hydrolyzed by boiling with 50% hydrochloric acid for 24 hours. After removal of the acid under reduced pressure, the hydrolysate was also standardized to contain 10 mg of total nitrogen per milliliter.

Standardization of the urine permits comparison of urine specimens from period to period and from patient to patient. The concentration selected yields chromatograms of satisfactory color intensity and insures detection of most of the amino acids present in the specimen. Concentration of normal urines to contain more than 40 mg of total nitrogen per milliliter prevents adequate separation since salts and other substances interfere. Removal of salt by common ion effect precipitation, impregnation of the filter paper and saturation of the solvents with salt has been unsatisfactory.

A two-dimensional chromatogram on Whatman No. 1 filter paper was prepared from each of the samples(1). One hundred microliters of the standardized solution were applied to the paper, and 25 microliters of 30% hydrogen peroxide were superimposed on the spot to effect quantitative oxidation of cystine and methionine to cysteic acid and

\* Funds to support this investigation were provided by a grant from the Donner Foundation, Inc.

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TABLE I.  
Effect of Varying Food and Fluid Intake on Rate of Lymph Flow.

Diet	Fluid intake	No. of rats	Avg BW (g)	Time of collection, (hr)	Lymph flow (ml)	Rate of flow (ml/hr/100 g)
Fasting	Water	4	160* (110-250)	29* (18-47)	11.4* (7.3-18.6)	0.26* (0.12-0.38)
"	1% NaCl	6	250 (145-320)	25.5 (22-30)	29 (15-61)	0.48 (0.16-0.98)
Fed	Water	4	229 (212-240)	105 (93-118)	106 (64-163)	0.44 (0.29-0.62)
"	1% NaCl	3	280 (170-315)	124 (111-140)	727 (657-784)	2.45 (1.98-3.29)
"	1% NaCl	9	239 (183-386)	52 (40-72)	165 (137-302)	1.45 (0.88-2.2)
Fed†	Water	5	153 (130-168)	43 (20-72)	60 (13-123)	0.86 (0.39-1.21)
	1% NaCl			44 (27-72)	216 (57-457)	3.3 (1.5-4.7)

\* Average value for the group. Figures in parentheses indicate ranges.

† The animals in this group were maintained alternately for 24 hr periods on 1% NaCl solution and water *ad lib*.

TABLE II.  
Average Daily Output of Lymph in Fed Rats Maintained on Orally Ingested Water or 1% NaCl Solution.

Fluid intake	No. of rats	Avg BW (g)	Avg vol. (ml) of lymph per consecutive day				
			1	2	3	4	5
Water	4	229	9	31	27	21	18
1% NaCl	3	280	28	76	125	138	186

stance (not recorded in table) 302 cc of lymph was collected in 23 hours from a rat weighing 145 g.

*Discussion.* The experiments reported here indicate that the fluid intake of the rat rises remarkably in the presence of a lymph fistula, if the animal ingests added sodium chloride. The control rat with a thoracic duct fistula is apparently able to make up for the loss in nutrients and electrolytes by establishing a dietary and fluid intake which is satisfactory for the maintenance of a temporary equilibrium. If, however, sodium chloride is added to the diet, the requirement for fluid intake rises considerably. The abrupt increase in intake of saline drinking solution suggests that the animal is vigorously attempting to repair loss of fluids and electrolytes via the lymph fistula.

It is well known that the intravenous injection of large amounts of crystalloids will produce an increased lymph flow from the thoracic duct. The explanation for this effect(3) is that an hydremic plethora results, with consequent lowering of plasma colloidal osmotic pressure and increased capillary hydrostatic pressure. There is, then, a resultant loss of fluid to the tissue spaces, with consequent increase in lymph flow. This does not, however, explain the reason for the greatly increased *voluntary intake* of 1% NaCl solution and resultant lymph output as seen in the experiments cited here.

*Summary.* 1. A comparison is made of the

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TABLE I.  
Free Amino Acids in Unhydrolyzed Urine.

Amino acid	Methods		
	Paper partition chromatography		Microbiological assay (4, 11, 12, 15)
	10 mg total N per ml	34 mg total N per ml	
—alanine	+	+	0
„	—	+	0
Arginine	—	—	+
Cystine	+	+	+
Glutamine	+	+	—
Glycine	+	+	+
Histidine	+	+	+
Isoleucine	—	*	+
Leucine	—	+	+
Lysine	—	—	+
Methionine	—	—	+
Phenylalanine	—	*	+
Proline	—	—	+
Serine	+	+	+
Threonine	—	+	+
Tryptophane	—	*	+
Tyrosine	—	+	+
Valine	—	+	+
Total No.	6	11	15

\* May be obscured by leucine.

+ Reported as usually present.

— Not reported as usually present.

0 No information available.

Certain of the amino acids are excreted mainly in the combined form. Ninety-nine percent of aspartic acid, 90% of glutamic acid, 80% of proline, 77% of valine, and 71% of isoleucine, for example, are known to be excreted in the urine in peptide combination or as conjugates.(15) More lysine, threonine, leucine, and arginine have also been found to be excreted in the bound form than in the free state.(12) Harvey and Horwitt(6) concluded that not all of the free amino acid determinations by microbiological assay yield valid results because of the inhibiting effect of urea and other substances in urine.

As the sensitivities of paper partition chromatography and microbiological assay are comparable,(3,8,9) the discrepancy in results suggests that the microbiological procedure may detect other than free amino acids in

urine. Peptides or similar substances excreted in urine may not yield a color after reaction with ninhydrin and hence are not detectable on chromatograms, but their component amino acids apparently are available to the micro-organisms. The growth-promoting action of certain peptides has been demonstrated with some strains of micro-organisms,(10) and utilization by other strains may yet be demonstrated.

**Summary** The number of free amino acids found in unhydrolyzed urine by paper partition chromatography is less than that indicated by microbiological assay. No discrepancy is apparent between the methods when hydrolyzed urines are analyzed. As the sensitivities of the two methods are comparable, it is suggested that microbiological techniques are not as specific as the chromatographic procedure for the detection of urinary amino acids in the uncombined state.

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10. Simmonds, S., and Fruton, J. S., *Science*, 1949, v109, 561.

methionine sulfone, respectively, which appear as discrete spots on the developed chromatogram. Without such conversion, cystine is destroyed and methionine is obscured by leucine, isoleucine, tryptophane, and phenylalanine. Phenol and a mixture of equal parts of  $\gamma$ -collidine and 2,4-lutidine were the solvents regularly employed.

Each spot on the chromatogram was outlined in pencil as soon as possible after color had been developed with 0.1% triketohydrindene hydrate (ninhydrin) in *n*-butanol to insure that interpretation was complete before the color faded. The amino acids and peptides were identified after calculation of the  $R_F$  values, by comparison with a control sheet to which standardized solutions of the amino acids had been applied, or by reference to a map of the spots similar to the one prepared by Dent.(3)

Other ninhydrin-reacting substances than  $\alpha$ -amino acids(7) have seldom been found in urine in sufficient concentration to cause confusion or interference. The identity of the spots which comprise the chromatogram is frequently verified to insure that known spots are not masking the presence of other substances. Glycine, for example, may completely obscure asparagine, and  $\beta$ -alanine may conceal glutamine. Visual identification of certain spots may be difficult because of their close proximity and identical color as in the case of leucine, isoleucine, phenylalanine, and tryptophane.

Chromatograms of standardized unhydrolyzed urines from a number of normal subjects generally reveal the presence of alanine, cystine, glutamine, glycine, histidine, and serine. Three-fold concentration of an aliquot has revealed only  $\beta$ -alanine, leucine, threonine, tyrosine, and valine in addition. Although glutamic acid may be present, it has been demonstrated in this laboratory that it represents, in part at least, the amide which has undergone hydrolysis during storage or when exposed to the solvents during the run. Glutathione is also seen, as well as "over-glycine",

often called taurine in the literature.(3) Doubt exists that "over-glycine" is in fact taurine, however. The infra-red absorption spectrum obtained from a sample of "over-glycine" washed from undeveloped chromatograms after localization differs from that of a standardized taurine solution.(5)

Dent, utilizing the same technique, has reported that normal urines usually contain glycine and alanine.(2) Glutamic acid and valine occasionally appear, and  $\alpha$ -amino-n-butyric acid, citrulline, histidine, leucine, serine, and tyrosine may be found. His specimens, however, were not standardized to constant nitrogen concentration and usually contained less  $\alpha$ -amino nitrogen than the samples analyzed in this investigation.

Arginine, cystine, glycine, histidine, leucine, methionine, phenylalanine, serine, threonine, tryptophane, tyrosine, and valine are found regularly by microbiological assay in an allegedly uncombined state in the urine of normal human subjects.(4,11,12,15) Proline, lysine, and isoleucine are sometimes present, too. Aspartic acid is detected infrequently. Free glutamic acid is found only when precautions have been inadequate for preservation of the specimens.(4) The amino acids detected in normal, unhydrolyzed urines by both methods are compared in Table I.

Determination of the amino acids in hydrolyzed urines by both microbiological and chromatographic methods yields comparable results. Whether measured microbiologically or chromatographically, hydrolysis results in an increase in the number and amount of amino acids over those found in the free state. (4,11,12,15,16)

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TABLE I.  
Protocol for Aspartic Acid Deaminase.

	No. 1, ml	No. 2, ml	No. 3, ml	No. 4, ml
Bacterial suspension in pH 4 M phosphate buffer	0.5	0.5	0.5	0.5
Na <sub>2</sub> PO <sub>4</sub> (saturated)	0.5	—	—	—
Aspartic acid* in Na <sub>2</sub> PO <sub>4</sub> (saturated)	—	0.5	0.5	0.5
H <sub>2</sub> O	1.0	1.0	—	—
Biotin†	—	—	1.0	—
Yeast extract‡	—	—	—	1.0

\* 266 mg dl-aspartic acid dissolved in 100 ml saturated Na<sub>2</sub>PO<sub>4</sub>. Calculated to give 0.005 M dl-aspartic acid in the final reaction mixture.

† 0.0001  $\gamma$  or 0.001  $\gamma$  d-biotin/ml.

‡ 0.1 mg or 1.0 mg yeast extract/ml.

TABLE II.  
Summary of Aspartic Acid Deaminase Experiments.

Exp. No.	Organism*	pH of deaminase reaction	Ammonia nitrogen formed		
			No supplement, $\gamma$	Biotin, $\gamma$	Yeast extr., $\gamma$ †
1	<i>Proteus vulgaris</i>	7	11.7	15.8 (0.001 $\gamma$ )	15.1 (1 mg)
2	" "	7	7.0	14.8 (0.001 $\gamma$ )	13.7 (1 mg)
3	" "	7	4.3	15.9 (0.001 $\gamma$ )	17.3 (1 mg)
4	" "	7	2.3	11.3 (0.0001 $\gamma$ )	2.3 (0.1 mg)
5	" "	7	2.3	16.1 (0.0001 $\gamma$ )	5.4 (0.1 mg)
6	" "	4	0.0	0.0 (0.001 $\gamma$ )	0.0 (1 mg)
7	" "	4	0.6	0.6 (0.001 $\gamma$ )	0.0 (1 mg)
8	" "	4	0.1	0.5 (0.0001 $\gamma$ )	1.0 (0.1 mg)
9	<i>Escherichia coli</i> (Gratia)	4	0.0	0.0 (0.001 $\gamma$ )	0.0 (1.4 mg)
10	<i>Escherichia coli</i> (Gratia)	4	3.0	3.0 (0.001 $\gamma$ )	0.0 (1.4 mg)

\* All bacterial preparations were washed with water and inactivated by exposure for 30 minutes at 37°C to M phosphate buffer of pH 4 except in experiments 7, 9 and 10, where inactivation was at 25°C, and in experiment 10, where in addition to inactivation at the lower temperature the cells also were unwashed.

† 1 mg of yeast extract contains after vigorous acid hydrolysis prior to microbiological assay about 0.001  $\gamma$  of biotin.

unsuccessful. When the amounts of biotin and yeast extract used to stimulate deamination at pH 7 are titrated downward, contrary to Lichstein who worked at pH 4, biotin was

found to be more active in the system than is an equivalent of biotin as it occurs in yeast extract.

Despite the fact that inactivation of bac-

## Biotin in the Bacterial Deamination of Certain Amino Acids. (17496)

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Lichstein and coworkers(1,2,3,4) have reported that the aspartic acid, serine, and threonine deaminase activity of certain bacteria may be inactivated by exposure of the cells for a short time to pH 4 in M phosphate buffer. In their experiments the deaminase activity of such inactivated cells could be restored with biotin. Subsequent investigators(5) have been unable with biotin to restore deaminase activity to bacterial cells similarly treated and the fundamental observation that biotin somehow functions in deaminase activity has remained unconfirmed by any other group. Although certain discrepancies exist between the results obtained by Lichstein and those found in this laboratory, the activation of treated cells with biotin under certain defined conditions has been obtained, thus furnishing corroborative evidence for the involvement of biotin in the bacterial deamination of certain amino acids.

**Procedure.** Eighteen-hour cultures of the organisms used were obtained following growth at 37°C on a medium composed of 1% yeast extract (Difco), 1% tryptone (Difco), 0.5% disodium hydrogen phosphate, and 0.1% sodium formate. The medium was dispensed in 100 ml quantities in 250 ml Erlenmeyer flasks. The pH before autoclaving at 120°C for 15 minutes was 7.35. Following growth the organisms were centrifuged down rapidly (Servall angle centrifuge, plastic cups), resuspended in 50 ml of distilled water, and recentrifuged. The washed cells thus obtained were suspended in about 3 ml of M phosphate buffer of pH 4. Inactivation with respect to deaminase activity

was accomplished on aliquots of this suspension. These aliquots were dispensed in open test tubes that usually were immersed in a water bath at 37°C. Thirty minutes was the usual inactivation time. Immediately following inactivation of the cells the deaminase reaction was carried out at 37°C by appropriate additions to the same tubes. A protocol for deamination at pH 7 is given in Table I. When the deamination reaction was carried out at pH 4, M phosphate buffer of pH 4 replaced the Na<sub>3</sub>PO<sub>4</sub> solution and was used also as a solvent for the aspartic acid employed.

Following the deamination reaction at either pH enzymatic activity was stopped by the addition of 0.5 ml of 20% trichloroacetic acid to each tube. The tubes then were centrifuged until a completely clear supernatant was obtained. Ammonia formed in each tube was determined by adding to 2 ml of supernatant 2 ml of 10% NaOH and 1 ml of Nessler's reagent (gum ghatti stabilized). The color intensities were read in a Klett-Summerson colorimeter against a series of similarly prepared standard tubes of ammonium sulphate. The ammonia nitrogen found in tube 1 was subtracted from the results obtained in each other tube to give the ammonia nitrogen originating from the deamination of aspartic acid by the bacterial preparations used.

**Results.** The results obtained in a series of experiments are summarized in Table II. These data confirm those of Lichstein to the extent that they show that cells of *Protens vulgaris* or *Escherichia coli* can be obtained with a reduced ability to deaminate aspartic acid and that when the deaminase reaction is run at pH 7, after inactivation of the cells at pH 4 in M phosphate buffer, activity can be restored by either biotin or yeast extract. Attempts at reactivating cells with biotin when the deaminase reaction was run at pH 4 in M/2 phosphate buffer, such as described by Lichstein in his paper(3) demonstrating special activity for yeast extract, have been

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as determined colorimetrically by means of a photoelectric colorimeter with a 540 millimicron filter. The results were expressed in terms of the amount of dye bound by 400 mg of tissue, wet weight. In experiments with crystallized bovine plasma albumin (Armour) the protein was dissolved in M/15 phosphate buffer, pH 7.4, and used in a concentration of 5 g per 100 ml.

Tissues were denatured by the following methods (1) Heat: Tubes containing the tissue were placed in a bath of boiling water for 30 minutes; (2) Contact with urea in 6 Molar final concentration for three hours: The urea mixtures were placed in cellophane bags 30 cm in length and dialysed against distilled water for 5 days at 5°C to remove the denaturing agent. Aliquots containing 250 mg of tissue were used and the results were calculated on the basis of 400 mg of tissue. The effect of the tissues on the binding of PSP by albumin was determined by adding samples containing 400 mg of tissue to a mixture of 1.0 mg of PSP and 50 mg of albumin. The mixture was dialysed against M/15 phosphate buffer at pH 6.5. M/15 phosphate buffer was used for control of pH in all experiments and the pH of all solutions was determined with a glass electrode at the termination of each experiment. This buffer was found sufficient to maintain a constant pH during the course of the experiment. By mixing tissues with PSP and storing at 5°C for 48 hours with sampling at intervals it was found that the tissues employed did not cause a decrease in color of PSP.

*Results and Discussion.* The binding ability of liver, kidney, spleen, testis, pancreas and serum for PSP is shown in Table I. All of the tissues were found to bind the dye, although usually less than serum did; the liver was the most active tissue and pancreas the least. There was found to be some variation in the values obtained in the various experiments although the relative status of the tissues between themselves was maintained. The variation between the binding capacity of the liver of various rats was approximately  $\pm 25\%$ . No doubt this variation was due to the natural differences between

TABLE I.  
Binding of PSP by Native Tissues.

Tissue	No. of determinations	Range, units
Serum	6	60-104
Liver	10	30-87.5
Renal cortex	5	20.8-64
Renal medulla	5	29.4-60
Spleen	3	18.4-78
Testis	6	16.1-31.6
Pancreas	4	8-30

\* The units are micrograms of dye bound by 400 mg of tissue or 0.4 ml of serum.

TABLE II.  
Effect of Heat Denaturation on Binding of PSP by Serum and Tissues.

	Serum	Liver	Kidney	Pancreas
Unheated	62 (4)	65 (9)	44 (3)	30 (3)
Heated	8 (4)	125 (9)	75 (3)	55 (3)

Figures in brackets indicate the number of animals tested. Average values are given for micrograms of dye bound by 400 mg of tissue or 0.4 ml of serum.

the animals used. Denaturation of liver, kidney, and pancreas tissue proteins by heat or urea (Table II) resulted in a striking increase in the binding of PSP by all of these tissues. The increase in the binding by the liver tissue proteins denatured by both agents was of the same magnitude. In contrast to the tissues, denaturation of serum by heat markedly decreased its capacity for dye binding. The effect of denaturation of rat tissue proteins on dye binding is similar to the results obtained with egg albumin. These facts suggest that, in the denaturation process, either more foci of attachment for the PSP are opened in the binding proteins, as is the case with egg albumin, or that hitherto inactive proteins have become capable of binding.

When homogenates of native liver, kidney or pancreas were added to bovine albumin (Fig. 1) the binding of PSP was much less than the arithmetic sum of the dye bound by albumin alone plus the dye bound by the tissues alone. On the other hand no such decrease of binding was observed when heat-denatured tissue homogenates were added to bovine albumin; the mixture bound nearly as much PSP as the proteins did when tested separately. This experiment is interpreted as indicating that binding occurred between the



terial deaminase by exposure to molar phosphate buffer at pH 4 is not yet understood, is not always consistent, and doubtlessly is influenced by a variety of factors, these data afford confirmation that biotin somehow is concerned in activation of the system. Further study of the variables involved during inactivation and reactivation of the deaminase system is indicated before final conclusions can be

drawn concerning the significance of biotin in these reactions.

*Summary.* Inactivation of bacterial aspartic acid deaminase activity by exposure of cells to pH 4 M phosphate buffer and its reactivation with biotin has been confirmed.

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### Binding of Phenolsulfonephthalein by Tissues and the Effect of Denaturation Thereon.\* (17497)

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The present experiments were conducted to study (1) the binding of the organic anionic dye phenolsulfonephthalein (PSP) by homogenates of several tissues of the rat; (2) the effect of denaturation of tissue proteins on this binding; and (3) the competitive effect of native and denatured tissues on the binding of PSP by crystallized bovine plasma albumin. It has been previously established that certain proteins can form complexes with organic anions on both the acid and basic side of the isoelectric point. De Haan(1) and Marshall and Vickers(2) demonstrated that the anion PSP was bound appreciably by serum proteins and Grollman(3) showed that at the pH of normal blood the binding was primarily by serum albumin, the globulins binding very little dye. Klotz and Urquhart(4) showed that anions are also bound to  $\beta$ -lactoglobulin, hemocyanin and  $\alpha_2$  globulin, although to a much smaller extent than occurs with serum albumin. Ohta(5) working with tissue brei preparations pre-

sented evidence that both anionic and cationic dyes were adsorbed by tissue proteins. Denaturation of serum albumin by heat(6,7) or urea(7) abolishes the binding capacity for oleate and methyl orange anions. A different effect occurs with egg albumin in that heat denaturation increases the binding of alkylbenzenesulfonate detergents(8) and methyl orange.(7)

*Experimental.* Albino rats were exsanguinated by cardiac puncture or decapitation and weighed samples of the tissues were homogenized for approximately two minutes in M/15 phosphate buffer, pH 6.5. In the experiments, measured volumes containing 250-500 mg of the original tissue together with 1.0 ml of a solution of PSP in phosphate buffer pH 7 were placed in a cellophane bag 15 cm long and 1.5 cm in diameter. The samples were dialyzed at 5°C against M/15 phosphate buffer pH 6.5 in flasks which were slowly rotated for 40 hours to reach equilibrium; the total volume of the system was 100 ml. The bound PSP was calculated from the concentration of free PSP in the dialysate

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diseases thought to be caused by herpes simplex such as Kaposi's Varicelliform Eruption,(3) dendritic ulcers of the cornea(4) and herpetic stomatitis(5) are being reported in increasing numbers. Because of the effectiveness of aureomycin against the larger viruses and the optimistic clinical reports against herpes simplex infections it seemed important to determine the *in vitro* effects of this antibiotic on the herpes simplex virus and to evaluate its *in vivo* action under the most favorable possible conditions in embryonated chick eggs.

**Method and Materials.** The toxicity of aureomycin was determined in chick embryos 10 to 12 days of age which averaged 50 g in weight. The eggs were prepared by the false air sac technique,(6) and the individual aureomycin dose dissolved in 0.2 ml of gelatin saline solution, was inoculated onto the "dropped" chorioallantois. The eggs were incubated at 96°F and 76% relative humidity. They were candled at least twice daily and death of the embryo was used as the end point. All dead eggs were cultured in Brewer thioglycollate medium for possible bacterial contamination. For drug controls penicillin in a concentration of 500 units/ml was used in some experiments and inactivated aureomycin in others. The aureomycin control was prepared by heating aureomycin at 56°C for 5 days, and was shown by bioassay to contain less than 0.1% of its original activity. Two lots of aureomycin were used. The first series of experiments was performed with batch A (Lederle lot No. unknown) and the later series with batch B (Lederle lot No. 7-9022B).

The virus used was egg adapted in this laboratory from the HF Rockefeller strain by 129 serial passages on the chorioallantoic membrane. The inoculum for each experiment

was prepared from virus maintained in this laboratory by frequent passage. Different passages, each of which had a titer of approximately  $10^3$  infectious units per ml were used for each experiment. This strain of virus when properly diluted kills 95-100% of the chick embryos between the 69th and 90th hours. Embryos which died with virus plus drug were subcultured in other eggs to establish the presence of virus.

The *in vitro* effect was determined by mixing the drug and 10% suspensions of the infected membranes; incubating at room temperature for 4 hours; diluting the mixture sufficiently to obviate any *in vivo* effect; and injecting the inoculum onto the chorioallantoic membrane. Penicillin (500 units/ml) which had previously been shown to have no virucidal action was used as control.(7) To determine an *in vivo* effect one experiment was performed in which the virus and drug were inoculated into the yolk sac, the virus follow-

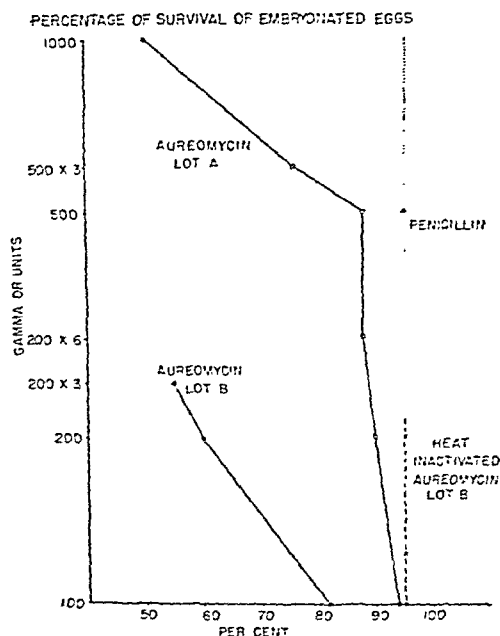


FIG. 1.

The toxicity of aureomycin and penicillin for embryonated eggs. The drug was inoculated onto the chorioallantoic membrane and death within 60 hours was taken as end point. Most points are the results from 24 or more eggs.

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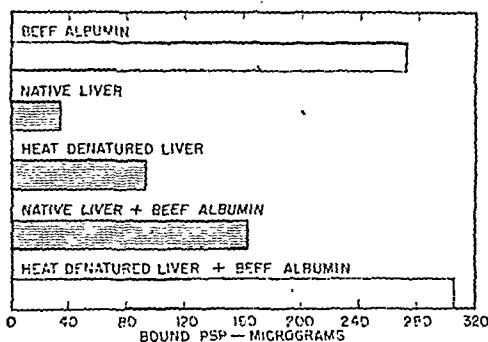


FIG. 1.

Effect of adding native, and heat denatured, homogenized tissue on the binding of phenolsulfonephthalein by crystallized bovine plasma albumin, 50 mg.

tissue proteins with the albumin molecule thus decreasing the available points of attachment for PSP in the system while heat denaturation of the tissue proteins decreased

their attraction for albumin increasing the amount of dye anions bound by the mixture.

**Summary.** 1. Tissue proteins are capable of binding the anionic dye, phenolsulfonephthalein, in small amounts. All of the tissues examined bound less of the dye than did serum and different tissues appear to have different capabilities in this respect. Of the tissues tested liver bound most and pancreas least.

2. Denaturation of tissue proteins by heat or urea materially increased the ability of the tissue protein to bind the dye.

3. Native tissue proteins of the rat considerably decreased the binding of phenolsulfonephthalein by crystallized bovine albumin; this property was abolished by heat denaturation of the tissue proteins.

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### Effect of Aureomycin on the Herpes Simplex Virus in Embryonated Eggs.\* (17498)

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Aureomycin was shown by Wong and Cox to have marked therapeutic activity against the viruses of the psittacosis-lymphogranuloma group.(1) They demonstrated that 49 of 55 treated chick embryos survived experimental infection with psittacosis through the 13th day, contrasted with survival of only 3 of 57 control embryos. These results were found to be essentially true for the other

members of the psittacosis-lymphogranuloma group (lymphogranuloma venereum, psittacosis, SF strain human pneumonitis, mouse pneumonitis, feline pneumonitis, and meningopneumonitis F97 strain) as well as for the rickettsiae. In contrast with the marked activity *in vivo*, they conclude that the drug had no *in vitro* effect against these agents. Harned *et al.*, in a study on the acute toxicity of intravenously administered aureomycin hydrochloride for several laboratory animals, found the LD50 for the drug to be 134 mg/kg for mice and 118 mg/kg for rats.(2) Clinical accounts of successful treatment of

\* This work was supported by a grant from the U. S. Public Health Service.

The aureomycin used in this study was supplied by Lederle Laboratories Division of American Cyanamid Company, New York City.

Much of the preliminary work incident to this study was done by Dr. Lewis L. Coriell.

The activity levels for inactivated aureomycin were performed by Dr. Coleman Whitlock.

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the second batch of aureomycin. Under the experimental conditions, aureomycin inactivated by heat, and penicillin were non-toxic. The LD50 of the least toxic lot of aureomycin for the chick embryo was 20 mg/kg. The *in vitro* effect of aureomycin on the herpes simplex virus is shown in Table I. The drug apparently has no *in vitro* action on the virus, under the conditions of the present experiments. The *in vivo* effect of aureomycin on the herpes simplex virus is shown in Table II. Because of the difference in toxicity and a possible difference in activity in the two lots of aureomycin, the results with each lot are recorded separately. There seems to be little difference in the effectiveness of the two lots of drug. Although in some of the dilutions small differences occurred between the controls and the drug treated embryos, at the critical dilutions with moderately large

doses of drug and small amounts of virus there is no significant increased survival of aureomycin treated eggs. Analysis of the data also failed to show any significant delay in time of death of treated infected eggs.

**Conclusions.** (1) Aureomycin appears to be more toxic for the chick embryo than for most other laboratory animals. (2) There seemed to be a significant difference in toxicity between the 2 lots of aureomycin used. Incubation of aureomycin at 56°C for five days destroys the embryo-toxic principle as well as its antibiotic activity. (3) Aureomycin has no *in vitro* activity against herpes simplex virus. (4) As compared with its activity against the larger viruses, no similar *in vivo* effect against herpes simplex virus could be demonstrated.

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### Remission of Disseminated Lupus Erythematosus Induced by Adrenocorticotropin.\* (17499)

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This report describes the effect of pituitary adrenocorticotrophic hormone (ACTH) in inducing 3 temporary dramatic remissions in 2 patients with classic disseminated lupus erythematosus. There was a virtually complete clinical remission while the ACTH was being administered. A description of this phenomenon has not been previously published.

Case I: A.F., a 36-year-old white housewife was admitted complaining of fever, weakness, weight loss, and arthralgia of 9 months' duration. She appeared acutely ill, with a daily temperature of 101°F. There were erythematous, scaly plaques on both cheeks which also showed telangiectatic vessels and areas of atrophy. The eruption, in addition,

involved the forehead, chin, ears, scalp, arms and legs. Scalp and pubic hair were sparse. There was a soft, blowing, systolic murmur over the 4th left intercostal space. The spleen was not palpable. Examination of the blood showed anemia, leukopenia, thrombocytopenia and an elevation of the sedimentation rate: protein and microscopic collections of red blood cells were constantly present in the urine. A section of the involved skin of the face showed changes usually seen in disseminated lupus erythematosus.

Beginning May 24, 1949, 150 mg of ACTH was injected intramuscularly in 6 divided doses daily for 5 days when they had to be stopped because further supplies of this scarce hormone were unavailable.

A remarkable change in the patient's condition was apparent within 12 hours after the injections were started. She appeared more

\* The assistance and cooperation of Dr. Dale Doherty and Dr. S. Biloon in the clinical study of these cases is appreciated.

TABLE I.  
*In vitro* Effect of Aureomycin on Herpes Simplex Virus.

No. of infectious units inoculated into each egg	Cone. of aureomycin in <i>vitro</i> before dilution and inoculation	Amt. of aureomycin inoculated into each egg	Mortality of eggs inoculated with aureomycin- treated virus	Mortality of eggs inoculated with control (penicillin)- treated virus
$5 \times 10^5$	4000 $\gamma$ /ml	2 $\gamma$	12/12	12/12
$5 \times 10^4$	4000 $\gamma$ /ml	0.2 $\gamma$	10/10	12/12
$5 \times 10^3$	4000 $\gamma$ /ml	0.02 $\gamma$	12/12	12/12

ing the drug by 30 minutes. To detect minimal effects and insure maximal contact of drug with virus, additional experiments were done on the chorioallantoic membrane with the technique of inoculation as described in the toxicity studies. In one experiment the virus preceded the drug but in the others the drug was inoculated 30 minutes before the virus. In addition, in several experiments the drug was reinoculated at 12-hour intervals for a total of 3 or 6 doses.

**Results.** The toxicity of aureomycin for embryonated eggs is indicated in Fig. 1. The two batches of aureomycin, which were obtained at different times showed an appreciable difference in toxicity. Because of this, experiments to determine the effect of the larger doses on the virus could not be repeated with

TABLE II.  
Mortality of Embryonated Eggs Inoculated on the Chorioallantois with Drugs and Then with Herpes Simplex Virus.

Approx. No. of infectious units inoculated	Aureomycin batch A						Aureomycin batch B (No. 7-9022 B)					
	100 $\gamma$ /egg		200 $\gamma$ /egg		500 $\gamma \times 3$ /egg		100 $\gamma$ /egg		200 $\gamma$ /egg		200 $\gamma \times 3$ /egg	
	Aureomycin	Control	Aureomycin	Control	Aureomycin	Control	Aureomycin	Control	Aureomycin	Control	Aureomycin	Control
$5 \times 10^5$	20/21	22/22	17/23	27/27	4/10	13/13	8/8	9/9	7/7	10/10	14/14	18/18
$5 \times 10^4$	19/21	21/22	18/26	25/27	5/13	12/12	7/8	10/10	8/9	10/10	7/12	14/18
$5 \times 10^3$	16/22	15/22	16/25	20/26	1/9	4/12	6/8	10/10	2/3	10/10	4/7	10/17
$5 \times 10^2$							4/9	5/10	2/5	5/10		
$5 \times 10^1$							3/9	2/10	1/10	2/10		

likely that the hormones only inhibit or mask the peripheral manifestations of rheumatoid arthritis, rheumatic fever, lupus erythematosus and psoriasis and do not affect the underlying causative disease mechanism, since a clinical relapse usually occurs promptly after cessation of hormone treatment.(2,4) In acute gouty arthritis, on the contrary, ACTH apparently completely aborts the acute attack.(1)

There are none of the usual signs or symptoms of adrenal insufficiency in the diseases benefitted by ACTH or Compound E and it is unlikely that the hormones act as substitution therapy for a deficiency. The expected change in the ketosteroids, eosinophils, lymphocytes, and total white count is evidence for adequate adrenal cortical function.(6) A "pharmacologic" extension of the usual physiologic properties of the hormones may be responsible for their action. In this connection, it is interesting to note the similarities between the effects produced by the salicylates and the adrenal hormone. Both groups of compounds lower fever and sedimentation

rate, inhibit hyaluronidase,(7,8) cause hyperglycemia,(6,8) increase uric acid excretion(9,10) and are local anesthetics.(8,11) In addition, the salicylates and the adrenal hormones are both effective in rheumatoid arthritis and lupus erythematosus.(8)

*Summary.* Two patients with classical disseminated lupus erythematosus were given three separate courses of injections of adrenocorticotrophic hormone (ACTH). In each case, there was temporary, striking clinical improvement and fading of the skin lesions, accompanied by the expected signs of increased adrenal cortical activity. These results suggest that continued administration of ACTH or Compound E may be of benefit in lupus erythematosus.

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## A Test of Triazolopyrimidines on Mouse Sarcoma 180.\* (17500)

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For nearly two years the major portion of a cancer chemotherapy program(1) has been devoted to a study of compounds that might

serve as anti-metabolites to nucleic acid components. The studies were stimulated by the development of knowledge concerning the effects of anti-metabolites in bacteria and

\* This research was supported by funds from a grant of the American Cancer Society to the Memorial Hospital and from the Charles F. Kettering Foundation to the Wellcome Research Laboratories.

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alert, sat up in bed and expressed a desire for food for the first time in months. The arthralgia and myalgia disappeared. Within 24 hours, the temperature returned to normal and remained so as long as the hormone was given. The skin lesions began to fade and had almost disappeared by the 7th day after ACTH was started. The improvement in the appearance of the skin lesions continued for several days after the hormone was stopped, although the temperature was elevated on the day after ACTH was discontinued; thereafter the patient gradually relapsed into the clinical condition described before treatment was instituted. During the period of administration of ACTH, the eosinophils disappeared from the peripheral blood, the leukocyte count increased from 6,000 to 11,000, and the lymphocytes dropped from 48% to 23%. There was no appreciable change in the sedimentation rate or urinary findings. The urinary 17-ketosteroids increased from 5.2 mg to 27 mg. By June 11th the patient was irrational and appeared moribund. On the following day 150 mg of ACTH was again given daily and maintained for 3 days during which the skin lesions began to fade, she became rational, fever was partly controlled and appetite returned. On June 15th, when the supply of ACTH was exhausted, she again became irrational and died 4 days later.

Case II: S.P., a 30-year-old white housewife was admitted complaining of swollen joints and an eruption on the face of 6 months duration. The eruption was made worse by exposure to sunlight. The classical erythematous lesions were present upon the face; warm, painful swellings existed over the wrists and metacarpophalangeal joints of both hands and there was a daily rise of temperature to 101°F. The spleen was moderately enlarged. Marked weakness of the upper and lower extremities was regarded by a neurological consultant as the result of peripheral neuritis. Examination of the blood showed anemia, leukopenia and an elevation of the sedimentation rate. There was only an occasional trace of protein in the urine from which porphyrin was also absent. The histopathologic picture of the erythematous skin was that usually seen in disseminated lupus erythematosus.

Beginning June 5, 1949, 100 mg of ACTH was injected intramuscularly in 4 divided doses daily for 5 days. Striking improvement followed and was manifested by disappearance of the swelling and pain in the wrists and joints of the hands, the fading of the skin lesions, diminution in size of the spleen, increase in muscular strength of the upper and lower extremities, return of temperature and appetite to normal; in addition, the eosinophils disappeared from the peripheral blood, the leukocyte count rose from 5,600 to 12,400 and the sedimentation rate returned to normal. Within 2 days after ACTH was discontinued the temperature began to rise, and 5 days later, there was a clinical relapse.

*Discussion.* After the first published demonstrations(1,2) that adrenal cortical activity induced either by administration of ACTH or the synthetic adrenal cortical steroid, 17-hydroxy-11-dehydrocorticosterone (Compound E of Kendall) was effective in alleviating acute nonbacterial arthritis, it appeared desirable to test these hormones in other members of the group of so-called "collagen diseases"(3) that also exhibit arthritis as part of the clinical picture. The effectiveness of Compound E in acute rheumatic fever has already been reported.(4) In addition, not only the arthritis, but cutaneous lesions of psoriasis disappear after the administration of ACTH(5) and return upon its discontinuance.

The temporary remissions induced by 5 days administration of ACTH represent an unequivocal, dramatic improvement in the course of a progressive, usually fatal disease. There is reason to expect that the continued administration of ACTH or Compound E would prolong the remission. It appears

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TABLE I.  
Results with Triazolopyrimidines Tested Against Sarcoma 180.

SK No.	Name	Dose, mg/K/day	No. of mice*	No. of deaths	Tumor† inhibition
1054	5,7-diamino-1-v-triazolo (d)pyrimidine	250	20	9	— to ±
1059	5-hydroxy-7-amino-1-v-triazolo (d)pyrimidine	100	15	8	±
1149	5,7-dihydroxy-1-v-triazolo (d)pyrimidine	1500	15	8	— to ±
1150	5-amino-7-hydroxy-1-v-triazolo (d)pyrimidine	250	35	7	— to ±
1315	7-amino-1-v-triazolo (d)pyrimidine	125	35	23	— to ±

\* A total of 410 treated animals were used in test groups of 5 mice each. Data for only the highest doses are tabulated. The remainder of the mice were tested at lower levels of the compounds as follows: SK 1054, 128-200 mg/K/day, 60 mice; SK 1059, 16-70 mg, 65 mice; SK 1149, 500-1000 mg, 55 mice; SK 1150, 64-200 mg, 85 mice; SK 1315, 63-100 mg, 25 mice. Two or more preparations of each compound were used.

† The grading of tumor inhibition is described in the section on Materials and Methods.

64-150 mg/K/day, were higher and initiated earlier (24 hours after tumor implantation) than in the experiments with adenocarcinoma EO 771 reported elsewhere. The preparations of 8-azaguanine varied in purity from one containing 40% of the guanine analog and 60% of the xanthine analog to several containing only one component as judged by a paper chromatogram.† The tumor inhibitory effects with the several samples of 8-azaguanine tested at 200 and 250 mg/K/day are considered insignificant because they were obtained at lethal levels. The analogs of isoguanine, 2,6-diaminopurine, adenine and xanthine also showed inconsistent inhibitory effects but only at lethal levels.

The failure of 8-azaguanine to inhibit selectively the growth of Sarcoma 180 is inconsistent with the concept that tumor cells in general possess a guanine metabolism comparable to that demonstrated for tetrahymena. (15) Sarcoma 180 would appear to be the first exception to the stated concept of "uniformity of various cancer tissues with respect to guanine metabolism". (15) The difficulties

in generalizations concerning purine metabolism are further demonstrated by the observation (17) that with C 57 black mice bearing adenocarcinoma EO 771 there is an incorporation of guanine into nucleic acids of both normal and tumor tissue whereas in normal rats no significant incorporation has been observed. (6,7)

Regardless of the negative results obtained with 8-azaguanine against Sarcoma 180, it has been considered essential to test the compound in a spectrum of tumors. The results of these tests to be reported shortly (18) will reveal whether Sarcoma 180 possesses a metabolism unique among tumors with respect to guanine or merely represents a difference in response of Sarcoma 180 and mammary adenocarcinoma EO 771 to certain compounds such as found previously with folic acid analogs. (13)

*Summary.* 8-Azaguanine and 4 other triazolopyrimidines at tolerated doses were without inhibitory effect on Sarcoma 180.

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† We are indebted to Dr. Aaron Bendich of the Protein Chemistry Division of Sloan-Kettering Institute for this analysis.

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animals(2-5) and the increasing information regarding details of nucleic acid metabolism.(6-8) Approximately 25 purines, 200 pyrimidines, 50 pteridines, 40 folic acid analogs and numerous miscellaneous heterocyclic compounds have been subjected to test. Among the compounds found to be most active in this program are those with anti-folic acid activity(9) and 2,6-diaminopurine(10) for the prolongation of survival of leukemic mice and some folic acid analogs for the inhibition of Sarcoma 180 and a few other tumors.(11-13) Compounds showing less inhibition of Sarcoma 180 in repeated tests have been found in each of the categories mentioned above. The reproducible, weak effects have been of interest in providing leads for study of related compounds.

A number of triazolo[*d*]pyrimidines (8-azapurines)(14) were among those which failed to show a satisfactory inhibitory effect in tolerated amounts against Sarcoma 180. The recent report(15) of the inhibitory action of 8-azaguanine (5-amino-7-hydroxy-1-*v*-triazolo[*d*]pyrimidine), also referred to as "guanazolo," on mouse mammary adenocarcinoma EO 771, on spontaneous mammary cancer in C3H mice, and on lymphoid leukemia in strain A mice redirected attention to our findings. The action of the guanine analog was interpreted by Kidder as indicating a similarity in guanine metabolism between *Tetrahymena gelii* and neoplastic tissues and, a "uniformity of various cancer tissues with respect to guanine metabolism".(15) An extension of our ex-

periments with 8-azaguanine, 8-aza-adenine, 8-aza-isoguanine, 8-aza-2, 6-diaminopurine and 8-azaxanthine against Sarcoma 180 under conditions favoring demonstration of inhibitory action confirmed the ineffectiveness of these purine analogs at safe dosages. Inhibition was observed with each compound only when it was given in amounts sufficient to kill a large percent of the mice.

*Materials and methods.* Samples of the triazolopyrimidines were prepared independently by two of the authors, (L.F.C.)(16) and (G.H.H.). Dr. M. L. Crossley of the Calco laboratories kindly supplied two additional samples of 8-azaguanine. The technic used in the Sarcoma 180 test has been described.(11) In brief, small tumor implants (1-2 mm in any dimension) were made subcutaneously by trocar into the axillary region of CFW or RF mice, 18-22 g in weight. Twenty-four hours later the compounds suspended in gum acacia were injected intraperitoneally twice daily for one week. The dosages used were based initially on maximum tolerated doses determined by Dr. F. S. Philips, Pharmacology Section. Higher toxic doses were subsequently used when inhibition was not found at the safe levels. At the end of the injection period, the tumors were measured with calipers in two diameters. The degree of inhibition of tumor growth was graded as follows:

*Marked inhibition (+)*, no growth of tumors in treated animals to a growth with an average diameter  $\frac{1}{4}$  that of the untreated controls;

*Slight inhibition ( $\pm$ )*, growth of tumors in treated animals from  $\frac{1}{4}$  to  $\frac{3}{4}$  the average diameter of the control tumors;

*No effect (-)*, growth of tumors greater than  $\frac{3}{4}$  the average diameter of the controls.

*Discussion of results.* The data are summarized in the table for only the highest levels tested with each compound. At lower toxic levels the compounds inhibited the tumors slightly and at non-toxic levels there was no effect. None of 8 samples of 8-azaguanine inhibited the development of Sarcoma 180, when tested at non-toxic levels. These levels.

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TABLE I.  
Effect of Homologous and Heterologous Spleen Pulp on Mitosis in Intestinal Epithelium.

Group	No. mice	Mitotic counts	
		Group avg $\pm$ S.D.	Range
I. Control <i>dba</i>	5	1.34 $\pm$ 0.250	(0.86-2.10)
II. Control Rockland	5	1.33 $\pm$ 0.267	(0.68-1.80)
III. Rockland spleen in Rockland	4	1.23 $\pm$ 0.295	(0.90-2.04)
IV. Rockland spleen in <i>dba</i>	5	1.55 $\pm$ 0.350	(0.82-2.46)
V. <i>dba</i> spleen in <i>dba</i>	2	1.21 $\pm$ 0.236	(0.80-1.74)

significance level 0.1%).

**Discussion.** The stimulation of mitotic activity obtained with heterologous tissue in these experiments tends to confirm the results of Breuhaus and McJunkin(4) using intraperitoneal injections of macerated rat kidney in normal and unilaterally nephrectomized rats.

Our results would indicate that a foreign tissue can influence mitotic activity as judged by the reaction of the epithelium in the crypts of Lieberkühn in the small intestine of the mouse, whereas a homologous tissue may not do so, as illustrated by the lack of significance of the data from *dba* mice receiving *dba* tissue. The borderline significance level for homologous tissue in Rockland mice, how-

ever, may possibly be attributed to the fact that this is a heterozygous strain and there would be a greater degree of difference between tissues in animals of this stock than between those in animals of the highly inbred *dba* line 1 strain.

**Summary.** The subcutaneous injection of spleen pulp from heterozygous Rockland mice into homozygous *dba* line 1 mice caused a significant increase in the mitotic activity in the epithelium of the crypts of Lieberkühn, whereas the injection of the spleen pulp from *dba* mice into mice of the same strain was without significant effect, and the injection of Rockland spleen into Rockland mice caused a mitotic decrease of borderline significance.

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## Choline Deficiency in the Hamster. (17502)

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In a previous communication(1) it was noted that there is a correlation between the occurrence of fatty livers in choline deficient animals and the ability of the livers of normal animals of a given species to oxidize choline. Thus, rats,(2), mice,(3) and dogs(4) develop

fatty livers on choline deficient diets and their livers possess an active choline oxidase.(5) In contrast, the guinea pig does not develop a fatty liver while living on a choline deficient diet(1) and the livers of this species are devoid of choline oxidase activity.(5) In the present work it has been found that the liver of the normal hamster shows only moderate choline oxidase activity and that liver fat accumulation in this species is less than that

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# Effect of Homologous and Heterologous Tissue on Mitosis in Heterozygous and Inbred Mice. (17501)

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(Introduced by Elton S. Cook)

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A significant inhibition of mitotic activity in the epithelial cells in the crypts of Lieberkühn in *dba* mice was produced by repeated subcutaneous injections of an aqueous alcohol-treated extract of beef spleen.(1) In the present experiments the effect of a single subcutaneous injection of splenic pulp of homologous and heterologous origin on mitotic activity of the cells in the crypts of Lieberkühn has been investigated in a highly inbred and a heterozygous strain of mice.

**Experimental.** Twelve *dba* line 1 mice from the Jackson Memorial Laboratories and 9 of the Rockland Farm all-purpose strain were employed in this study. The mice were approximately 6 to 8 weeks old and were allowed food and water *ad libitum*. Spleens were removed aseptically from donor mice of the same strains, pulped by passage through a fine meshed wire screen, and diluted 1:1 with Ringer's solution. A single subcutaneous injection of 0.5 cc was made into the groin of each experimental animal, and 7 days later the animals were killed, always at the same hour of the day in order to avoid introducing the diurnal variable in the mitotic cycle.(2) At autopsy a short segment of the small intestine located 20 mm distal to the pylorus was removed, fixed in Bouin's solution, dehydrated and embedded in paraffin. Histologic sections 8  $\mu$  in thickness were stained with hematoxylin.

Mitotic figures including those from late prophase to late anaphase were counted in the epithelium of the crypts of Lieberkühn in cross sections of the gut. Ten sections from progressive levels of the segment sufficiently separated to rule out counting the same figure more than once were used from

each animal, and 50 fields in each of these sections were studied at a magnification of 970, a total of 10,500 fields being explored.

Two untreated groups of animals consisting of 5 Rockland Farm (Group I) and 5 *dba* line 1 (Group II) mice served as controls. The effect of spleen pulp from the Rockland Farm mice on mitosis in 4 Rockland Farm mice (Group III) and 5 *dba* line 1 mice (Group IV) was studied, and also the effect of *dba* spleen pulp on mitosis in *dba* gut epithelium (Group V). There were not sufficient *dba* animals to study the effect of *dba* spleen pulp on mitosis in Rockland Farm mice.

The significance of the data obtained from the different groups was determined by the formula(3) designed for small samples:

$$t = \frac{M_1 - M_2}{\sqrt{\left( \frac{N_1 \sigma_1^2 + N_2 \sigma_2^2}{N_1 + N_2 - 2} \right) \left( \frac{N_1 + N_2}{N_1 N_2} \right)}}$$

Levels of significance are expressed in percentage.

**Results.** No significant difference in mitotic activity in normal control mice of the highly inbred *dba* strain and the heterozygous Rockland Farm mice was found (Groups I and II,  $t = 0.216$ ; significance level 20%). Homologous spleen in *dba* mice had no effect on the number of mitotic figures (Groups I and V,  $t = 1.97$ ; significance level  $> 20\%$ ) while homologous spleen in Rockland mice appeared to reduce the mitotic count slightly (Groups II and III,  $t = 1.67$ ; significance level  $< 5\%$ ). When splenic pulp from Rockland Farm mice was implanted in the *dba* mice, however, there was a marked increase in the number of mitotic figures observed (Groups I and IV,  $t = 3.42$ ;

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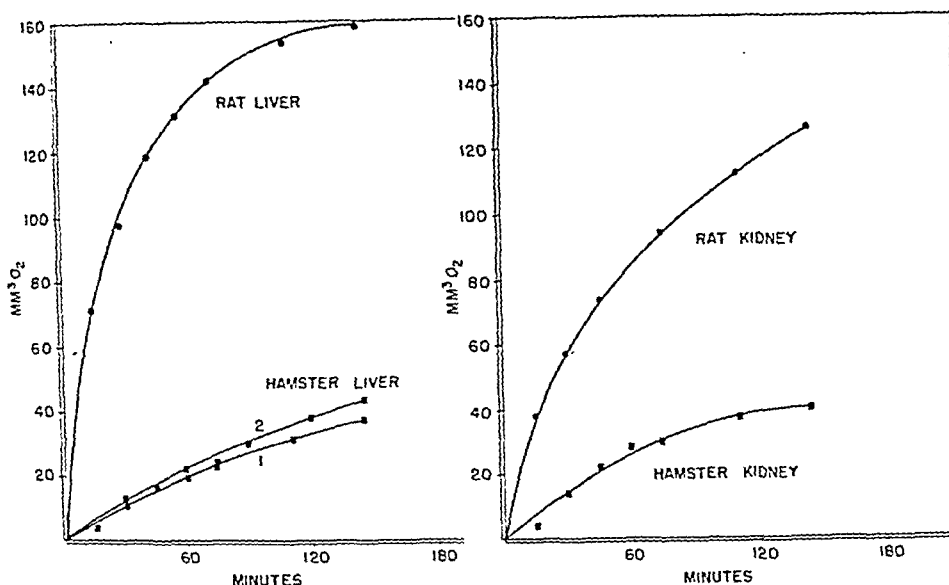


FIG. 1.

Choline oxidase activity of rat and hamster livers and kidneys. The substrate in each case was 1 mg of choline except for hamster liver where curves 1 and 2 represent the oxidation of 1 and 2 mg of choline, respectively. Theoretical oxygen uptake for 2 atoms of oxygen per choline molecule is 160 mm<sup>3</sup>.

were prepared by grinding in a mortar with sand and squeezing through muslin. Each Warburg vessel contained 0.5 ml of tissue suspension, diluted to 2 ml with M/20 phosphate buffer at pH 7.8. The choline chloride was dissolved in similar buffer and 1.0 mg of choline in 0.1 ml of buffer was added to the appropriate vessels at the expense of an equivalent volume of buffer. The vessels were then incubated at 37° in the conventional apparatus. The results are summarized in Fig. 1. In each case the control respiration in the absence of choline was subtracted before the data were plotted. It may be noteworthy that the basal respiration was of the same order of magnitude in rat and hamster. From the data shown in Fig. 1. it is apparent that hamster livers and kidneys do possess choline oxidase activity but this is markedly less than the activity of comparable rat tissues.

Because of the relatively poor growth and appetite of the hamsters on the peanut meal rations, as compared with the rats, it is not possible to determine whether the hamster is as susceptible to choline deficiency as the rat.

It remains true, however, that, to date, the livers of all species which develop fatty livers on choline deficient rations have been found to possess choline oxidase activity in greater or lesser degree.

*Summary.* Young hamsters develop fatty livers when fed choline deficient rations containing peanut meal, although the mean increment in liver fat is only half that found in rats on the same diet. This may be correlated with the finding that hamster livers and kidneys possess markedly less choline oxidase activity than do the livers and kidneys of rats or may be due solely to the relatively poor appetite and growth of hamsters on these rations.

The authors' thanks are due to the Nutrition Foundation and to the Duke University Research Council for their support of this work; to Merek and Company, Rahway, N. J., for the crystalline B vitamins employed; to the Wilson Laboratories, Chicago, Ill., for the liver fraction "L," and to the Lederle Laboratories, Pearl River, N.Y., for the folic acid.

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TABLE I.  
Composition of Diets.

	1 %	2 %
Casein	10.0	6.0
Salts <sup>*</sup>	5.0	5.0
Lard	20.0	25.0
Cotton oil	10.0	5.0
Sucrose	38.0	13.5
Cod liver oil	1.0	1.0
Cystine	0.3	0.3
Cholesterol	0.5	0.5
Inositol	0.1	0.1
Glycoeyamine	0.5	0.5
Peanut meal	—	30.0
Arginine	1.0	—
Liver "L"*	3.0	3.0

\* Wilson's Liver Fraction "L" obtained through the courtesy of Dr. C. E. Graham.

by decapitation and liver samples analyzed for total lipids by a method previously described.(6) The results of these experiments are summarized in Table II.

The failure to obtain fatty livers in the hamsters on choline-deficient diet 1 is referable to the apparent inadequacy of this "synthetic" ration for this species. Food consumption was lower than in the animals on diet 2 and during the course of the experiment there was a net loss in weight. Fatty livers are not to be expected under these circumstances despite the choline deficiency.(7-9) No explanation is at hand for the modest fat accumulation observed when diet 1 was sup-

TABLE II.  
Liver Lipids of Hamsters and Rats on Choline-Deficient Diets.

Group	Species	No. animals	Diet	Initial wt, g	Wt changes, g	Food intake, g/day	Liver lipids % wet wt
1	Hamster	6	1	96	-12	4.8	6.9
2	"	6	1 + choline	93	-7	5.2	9.4
3	Rat	6	1	100	23	6.1	13.9
4	"	5	1 + choline	97	25	6.3	4.9
5	Hamster	7	2	101	8	6.9	15.5
6	"	6	2 + choline*	102	7	6.7	7.5
7	"	7	2 + methionine†	97	7	7.1	5.7
8	Rat	6	2	103	17	6.8	24.7
9	"	5	2 + choline*				
10	"	6	2 + methionine†	99	23	6.9	7.6

\* Present as 0.6% of diet.

† Present as 0.8% of diet.

in rats on identical choline deficient rations.

**Experimental. Liver Fat Studies.** The animals were Syrian golden hamsters from a local colony of stock originally obtained from the General Biological Supply House, Chicago, Ill. They were housed in individual metal cages and offered the experimental rations *ad libitum* with water supplied at all times. The feeding technic was identical with that previously described for guinea pigs.(1) The basal diets are summarized in Table I. Each diet contained, in addition, per kilo of diet, thiamine 10 mg, riboflavin 10 mg, pyridoxine 10 mg, calcium pantothenate 50 mg, niacin 100 mg, *p*-aminobenzoic acid 50 mg, biotin 2 mg, folic acid 5 mg,  $\alpha$ -tocopherol 200 mg, 2-methyl-1, 4-naphthoquinone 10 mg. The feeding experiments were continued for 35 days. At this time the animals were sacrificed

plemented with choline. In contrast, all hamsters ingesting diet 2, which contained alcohol-extracted peanut meal were found to possess fatty livers and in the group shown in the table the lipid content varied from 11.9 to 19.1%. In another group of 14 hamsters fed the same diet, liver fat was found to vary from 9.4 to 28.1% with a mean of 15.3%. In general, there was a positive correlation between food consumption, growth rate and liver fat.

**Choline Oxidase Studies.** The animals were sacrificed by decapitation; liver and kidney

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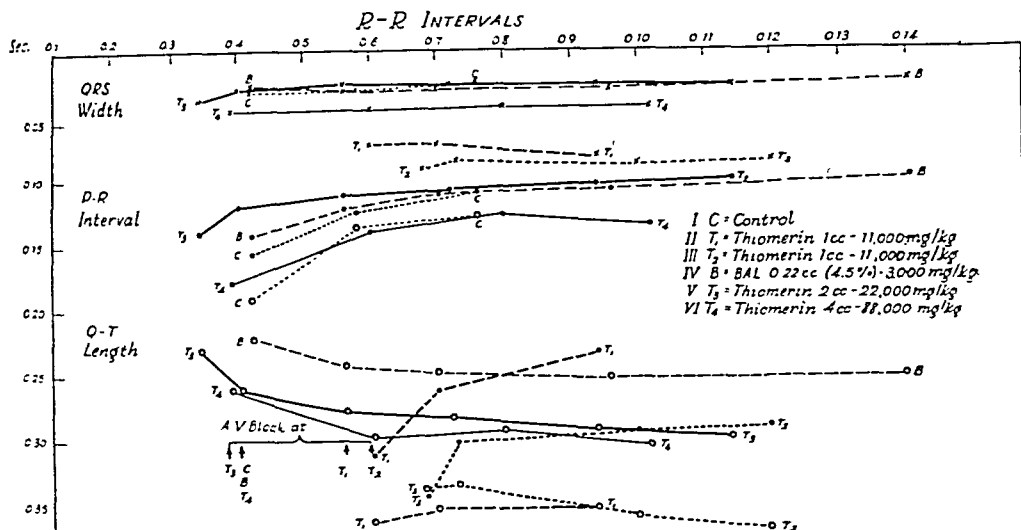


FIG. 1.

Example of usual reversal of thiomerin conduction effects by BAL, and BAL's cardioprotective value. Prolongations of P-R (dots), QRS (crosses), and Q-T (circles) intervals of the electrocardiogram at all rates of stimulation by thiomerin doses equivalent to 22,000 mg of mercury/kg—reversed by BAL. A total of 132,000 mg mercury equivalents per kg tolerated by the heart, with further deleterious but non-lethal conduction effects thereafter.

block, during the control and drug periods and analyzed by the magnifying Cambridge measuring instrument. P-R, QRS and Q-T intervals were then plotted against the R-R intervals for the control and other periods, as curves of recovery. Their prolongation or shortening at various rates of stimulation could thus be graphically evaluated. A total of 31 rabbit heart preparations were tested with thiomerin; 52 previous rabbit heart preparations provided comparative data for other mercurials.(7).

**Results. 1. Effects of thiomerin.** Thiomerin, like other mercurial compounds,(1,7) consistently and progressively prolonged conductivity in the ventricles, the atrioventricular conduction time, and the "electrical systole" or Q-T interval. Cardiac dilatation and slowing and lessened contractility resulted from large doses of thiomerin. It produced, to a much lesser extent than other mercurials,(7) monophasic type S-T segment displacements, doming and, rarely, reversals of the T waves. (Older batches of the drug were much more cardiotoxic, just as they showed greater ir-

ritation in the subcutaneous and intradermal tissues). Conduction effects were quite marked (prolongations of the QRS intervals of 50 to 150%) with doses of approximately 8,000 to 22,000 mg of mercury/kg of heart weight, with the new lots of the drug. (Older lots were similarly cardiotoxic in doses of 700 to 900 mg/kg). Rapidly (within 10 minutes) cardiolethal doses ranged from 54,000 to 69,000 mg of mercury/kg, with slow idioventricular rhythm and asystole occurring, to the exclusion of ventricular arrhythmias, as a terminal event. Transient ventricular ectopic beats and ventricular fibrillation occurred only once in 31 heart preparations after thiomerin and twice during control runs (rapid stimulation). The above figures indicate a comparative thiomerin cardiotoxicity of less than 1/200 that of mercurhydrin and 1/1,000 those of salyrgan-theophylline and mercuzanthin(7) in the isolated perfused rabbit heart. The cardiolethal ratios of thiomerin to mercurhydrin are less than 1 to 700, and of thiomerin to the other mercurials—1 to 1,800.

**Thiomerin and BAL.** The slight deleterious cardiac conduction effects of BAL in doses of 1800 to 3000 mg/kg have been previously dis-

# Cardiodepressive Effects of Thiomerin: Cardioprotective Attempts with BAL, Ascorbic Acid and Thiamin.\* (17503)

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The new mercurial diuretic, thiomerin, (mercaptomerin, N.N.R.) has been studied intensively by several groups of investigators,(1-5) since its introduction by Lehman.(6) His original experiments with cats indicated deleterious intraventricular conduction effects, representing toxicity less than 1/160 that of mercurial diuretics in current use which do not contain the monothiol component. There is evidence that in this mercaptide derivative the toxicity has been reduced without impairing the diuretic efficiency of the mercurial compound,(3) whereas the dithiol—BAL reverses both the toxic and diuretic effects of mercurials.(7) We have previously shown that BAL, ascorbic acid, and thiamin, in that order of efficacy, prevent and partially reverse the deleterious conduction effect of mercurhydrin (meralluride, N.N.R.) and other organic mercurials in the isolated rabbit heart.(8) The purposes of this study were (1) to obtain comparative cardiotoxic and cardiolethal dosage levels of thiomerin and (2) to determine the cardioprotective effects of BAL, ascorbic acid and

thiamin used before and after thiomerin in the isolated perfused rabbit heart.

**Methods.** The isolated rabbit hearts were perfused with a modified Locke solution at a constant temperature (37°C) and pH (7.4), and driven at definite varied rates with a thyatron stimulator, as in previous studies.(9) BAL, ascorbic acid, or thiamin, in predetermined amounts,(7) were injected into the tube leading to the aortic cannula either 10 minutes before or 10 minutes after thiomerin. Electrocardiograms corresponding to Lead I were obtained at various rates of stimulation, up to the production of A-V

TABLE I.

Doses of Thiomerin in mg/kg Heart Weight Producing Marked Conduction Delays (50% to 150% in QRS Width) and Rapid (Within 10 Minutes) Asystole in Isolated Perfused Rabbit Hearts.

	Conduction delays		
	mg/kg	(thiomerin	only)
8,100	"	"	"
8,300	"	"	"
9,200	"	"	"
11,000	"	"	"
11,200	"	"	"
13,500	"	"	"
14,000	"	"	"
14,600	"	"	"
15,400	"	"	"
15,800	"	"	"
17,300	"	"	"
17,700	"	"	"
22,200	"	"	"
Rapid asystole			
43,000	(after ascorbic acid)		
48,000	(after thiamin)		
54,000	(thiomerin only)		
56,000	(after thiamin)		
58,000	(after thiamin)		
61,000	(thiomerin only)		
62,000	(after ascorbic acid)		
68,000	(thiomerin only)		
69,300	(thiomerin only)		
72,000	(after ascorbic acid)		
77,700	(after thiamin)		
88,700	(after ascorbic acid)		
110,000	(after BAL)		
128,000	" "		
143,000	" "		
157,000	" "		

\* With the technical assistance of James E. Amburn. Supported in part by a grant from Campbell Products, Inc., New York.

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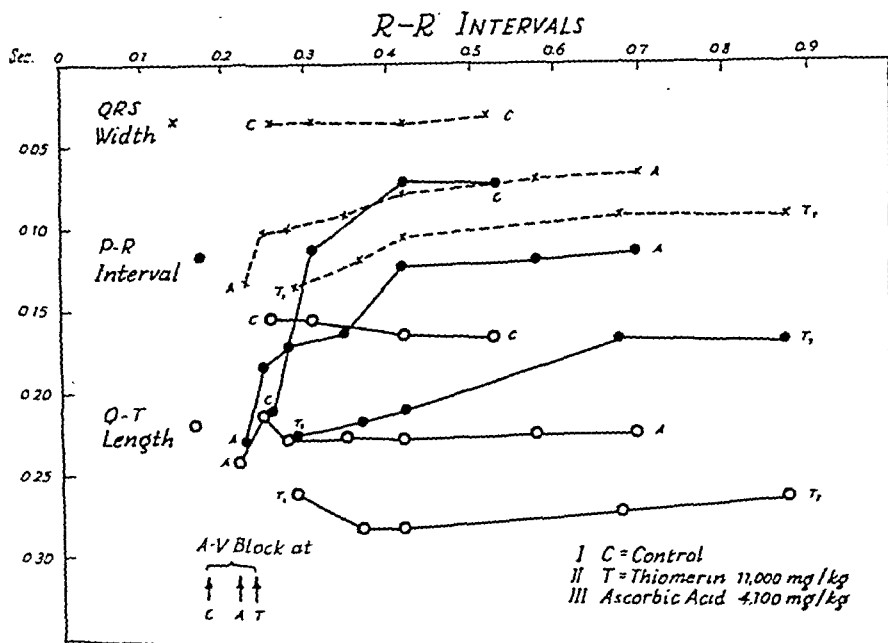


FIG. 3.

Example of frequent reversal of small dose thiomerin conduction effects by ascorbic acid. Prolongations of P-R, QRS and Q-T intervals of the electrocardiogram at all rates of stimulation by thiomerin are reversed, though less markedly than by BAL (Fig. 1), in all instances, by the large dose of ascorbic acid.

cardiac toxicity of less than 1/200 that of mercurhydrin. This ratio corresponds to Lehman's original figure of less than 1/160, measuring the comparative effects of the two drugs in prolonging the QRS interval in cats. Such marked innocuousness of thiomerin from the cardiotoxic standpoint is also emphasized by the absence of ventricular ectopic rhythms in the rabbit heart preparations, or in human subjects(3) given the drug subcutaneously and intravenously. Corresponding absence of marked subcutaneous and intracutaneous irritation both in animal and human subjects(10) of the newer lots of the drug is in marked contrast to the tender subcutaneous nodules and sterile abscesses, and erythematous, papular, vesicular and even necrotic intracutaneous reactions of other mercurials. It is of interest that whereas BAL, ascorbic acid and thiamin raised the maximum cardiolethal (causing asystole within 10 minutes) doses of meralluride to

above 10, 7.6 and 2.6 times the M.L.D. of the mercurial alone, respectively,(7) only BAL was successful in increasing the thiomerin tolerance, and that only to the extent of doubling the cardiolethal dosage. We can only speculate as to what the mercaptan linkage has to do with rendering thiomerin less toxic to the cardiac enzyme systems, and BAL of relatively little further cardioprotective effect. Work in progress in our neurochemistry laboratory indicates that oxidation is markedly suppressed or abolished for at least 30 minutes in rat heart slices by mercurhydrin.(11) The depression of respiration of yeast brought about by phenylmercuric nitrate is prevented by cysteine and homocysteine,(12) but more direct biochemical evidence relative to the cardioprotective effects of sulfhydryl compounds is not available. Likewise, the failure of ascorbic acid to in-

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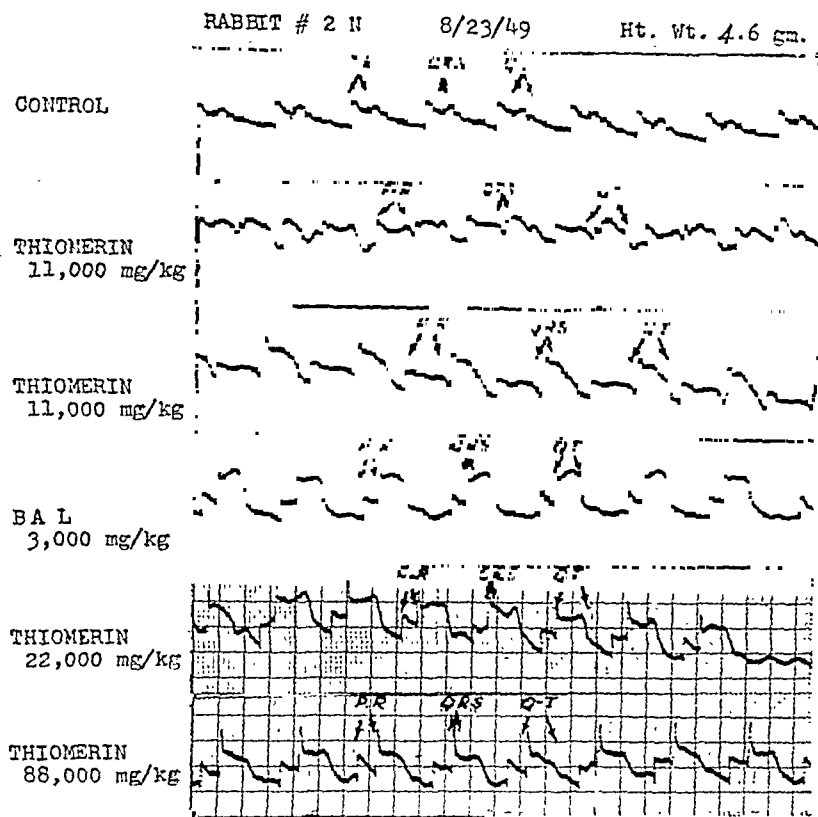


FIG. 2.

Electrocardiograms corresponding to lead I obtained at similar rates of stimulation during control, thiomerin, and BAL periods, 10 minutes apart, for same rabbit heart as in Fig. 1. The shortening of the P-R, QRS, and Q-T intervals by BAL, and their lengthening by thiomerin, much less after BAL than after control runs, are clearly seen.

cussed by us.(7) Thiomerin was tolerated thereafter in doses up to 157,000 mg of mercury/kg of heart weight (Table I), or approximately 2 times the usual cardiolethal dose, when preceded by BAL. BAL, likewise, markedly reversed the prolongation of the electrocardiographic intervals by previous injections of thiomerin into the closed perfusion system (Fig. 1 and 2).

*Thiomerin and ascorbic acid.* Ascorbic acid in doses of 4000 to 6000 mg/kg of heart weight caused slight deformations of the electrocardiographic patterns as previously reported.(7) In contrast to its action in increasing the cardiolethal dosages of mercurhydrin and the more toxic mercurials,(7) it had no such effect in respect to thiomerin (Table I). However, the slight conduction effects of small doses of thiomerin were often

partly reversed by ascorbic acid in the above dosage (Fig. 3).

*Thiomerin and thiamin.* Thiamin, in doses of 500 to 1000 mg/kg of heart weight caused slight changes in the electrocardiographic patterns as noted previously.(7) Again, in contrast to its effect on mercurhydrin, it failed to protect against more than the usual cardiolethal doses of thiomerin (Table I). Reversal of deleterious conduction changes resulting from small doses of thiomerin was the exception rather than the rule for thiamin (Fig. 4).

*Comment.* Samples of thiomerin received in the first half of the year 1949 showed cardio depressive effects corresponding to doses 20 to 25 times those of mercurhydrin (700 to 900 mg/kg vs. 20 to 36 mg/kg respectively). Newer lots of the drug, however, showed a

## Cardiodepressive Effects of Mercurial Diuretics. Cardioprotective Value of BAL, Ascorbic Acid and Thiamin.\* (17504)

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The use of mercurial diuretics is attended by cardiac abnormalities. The more serious, and even fatal, immediate reactions are ventricular fibrillation, preceded by ventricular tachycardia and ectopic beats, or, infrequently, cardiac asystole.(1) Elevation and other deformations of the S-T and T segments, notching of the QRS, prolongation of the P-R and QRS intervals up to alternating bundle branch block, auricular fibrillation and flutter, also occur, in human cases and in animal experiments.(1-3) BAL is conceded to be the most effective dithiol compound in combatting the toxicity of mercurial compounds, as well as neutralizing their diuretic effects.(4) Ascorbic acid likewise reputedly has antitoxic as well as synergistic diuretic effects when administered with mercurials.(1) Thiamin, reported beneficial in arsenical poisoning,(5) has not been used to counteract mercurial toxicity. Neither thiamin nor ascorbic acid have known direct cardiac effects, except that the T wave may become inverted after thiamin.(6) BAL has been observed to cause, beyond slowed cardiac rates, elevated S-T segments and deep S waves.(7)

The purposes of this study were to obtain

specific conductive and electrical systole effects of, particularly, mercurhydrin in the isolated rabbit heart; to compare its myocardial toxicity with that of other organic mercurials; and to evaluate the detoxifying effects of BAL, ascorbic acid and thiamin used before and after the mercurial compounds.

*Methods.* As in our previous investigations(8,9) with the isolated rabbit hearts, they were perfused per aorta and coronary arteries with a solution kept at 37°C, pH 7.4, and oxygenated with 5% CO<sub>2</sub> in oxygen. Electrocardiograms corresponding to lead I were obtained, as the auricles, or ventricles with the auricles cut off, were driven at various rates by means of a thyatron-type stimulator. The P-R, QRS and Q-T intervals were measured at various rates of stimulation with a Cambridge measuring instrument and graphed against the R-R intervals as curves of recovery (relative refractoriness). Control tracings were followed by the injections of the drugs in appropriate dosage, mercurials followed by the supposed antagonists, or vice versa. Ten minute intervals between doses of the same or different drugs allowed records of the maximum effects, and, together with rest periods between salvos of stimuli, eliminated the noxae of fatigue. The pH of the perfusate was not altered more than 0.5 unit by any of the drugs added, so that the acids (thiamin hydrochloride, ascorbic acid) administered needed no buffering. Moreover, our own studies(11) indicate that a pH range of 6.5-8.0 of the perfusate is unaccompanied by marked changes of the electrocardiographic intervals measured. A total of 52 experiments, 6 dealing with salyrgan-theo-

\* With the technical assistance of James E. Amburn. Supporter in part by a grant from Campbell Products, Inc., New York.

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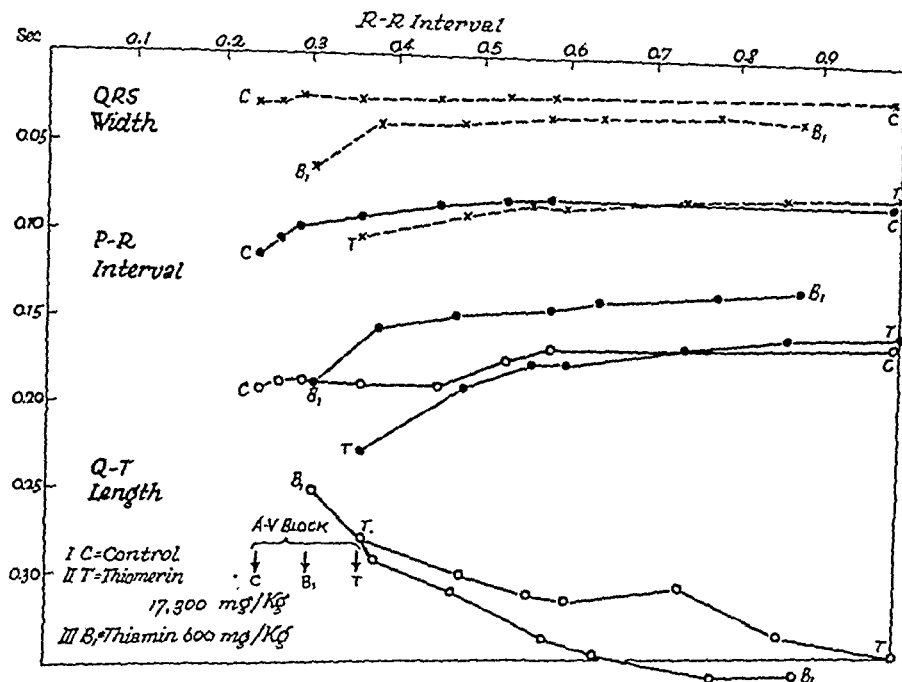


FIG. 4.

Example of infrequent reversal of small dose thiomerin conduction effects by thiamin. Prolongations of P-R and QRS intervals of the electrocardiogram at all rates of stimulation by a moderate dose of thiomerin are reversed by thiamin, as recorded for the three periods 10 minutes apart. The markedly prolonged Q-T intervals resulting from thiomerin are not shortened by thiamin. Compare similar results for mercurhydrin.<sup>7</sup>

crease further the cardiac tolerance of a mercurial compound with a mercaptide linkage may or may not be connected with possible thiomerin potentiation of the already present ascorbic acid in the cytochrome oxidase system in its active reductant state. This cannot be substantiated without further biochemical studies. For what obscure reason thiamin, and presumably its phosphorylated enzyme product — cocarboxylase, increases, though slightly, the cardiac tolerance to organic mercurial compounds other than thiomerin cannot be answered in the present state of our knowledge.

**Summary and conclusions.** 1. In the isolated rabbit heart thiomerin appeared to slow atrio-ventricular and intraventricular conduction and prolong the "electrical systole" (Q-T interval) in doses, as to mercury content, over 200 times those of meralluride, and 1000 or more times those of other commonly used mercurial diuretics. The rapidly cardiolethal dosages bore even larger ratios. Idio-

ventricular slowing and asystole, associated with marked cardiac dilatation and failure of contractility, and not ventricular ectopic rhythms, constituted the lethal manifestations of the thiomerin perfused heart.

2. In contrast to the marked increase in cardiac tolerance to other organic mercurial compounds as a result of preliminary administration of BAL, and to a lesser extent, of ascorbic acid and thiamin, BAL approximately doubled the cardiolethal dosage of thiomerin and the two vitamins were not significantly cardioprotective against the monothiol mercurial.

3. BAL markedly reversed the deleterious conduction effects of even large doses of thiomerin; ascorbic acid did so frequently, after relatively small doses; thiamin reversed the milder effects of thiomerin but in a minority of cases.



versal of A-V and intraventricular block, though the Q-T interval and the absolute refractory period were further lengthened (Fig. 2). In one instance of complete cardiac asystole after mercurhydriin, BAL caused resumption of idioventricular rhythm, though the heart failed to react to electrical stimulation. S-T changes of monophasic current type and doming or reversals of the T wave frequently followed the exhibition of either mercurials or BAL.

doses of mercurhydriin (36 mg of mercury/kg) caused, in general, partial reversal of A-V and possibly I-V block, and not infrequently further slight prolongation of the Q-T interval and the absolute refractory period of the recovery curves of conductivity (Fig. 4). The detoxifying effects were intermediate between those of BAL (supra) and thiamin (infra).

4. *Thiamin and mercurhydri*n. Thiamin in doses corresponding to therapeutic ones in man did not affect the electrocardiogram. Doses of thiamin of 570 to 780 mg/kg of heart weight at times impaired conductivity and prolonged the Q-T interval, with slight S-T and T changes. Mercurhydri

n administered after such amounts of thiamin was tolerated up to 196 mg/kg without killing the heart, although increasing block developed (Fig. 5). In one instance a mercuzanthin dose of 105 mg of mercury/kg similarly (Table I) produced A-V dissociation without immediate death of the heart after an apparently protective dose of thiamin (600 mg/kg). Thia-

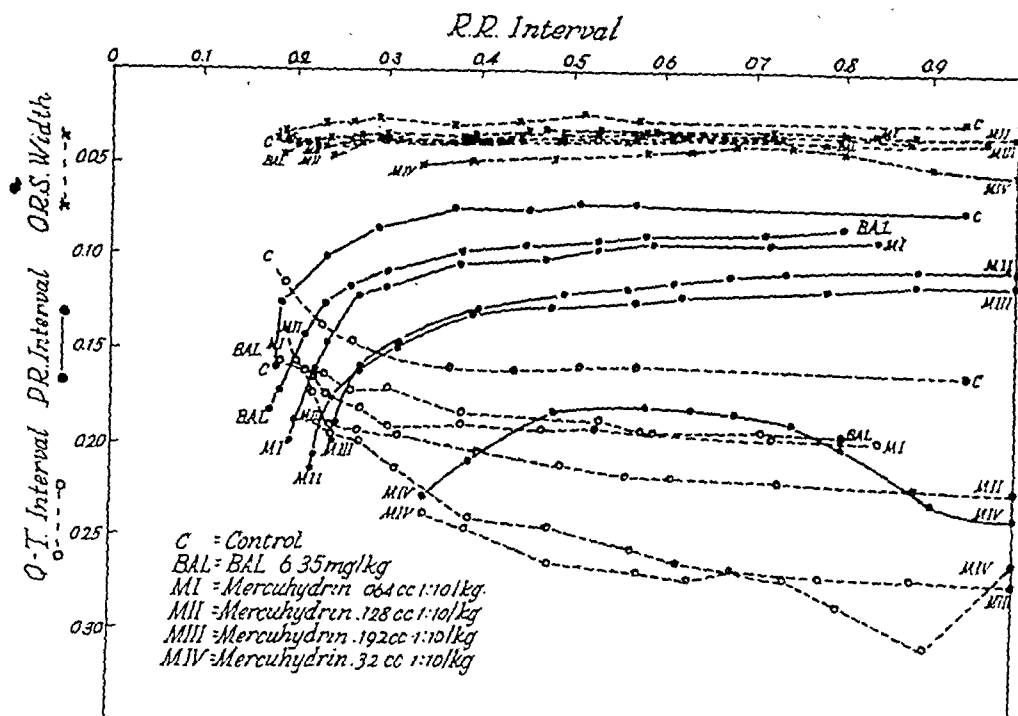


FIG. 1.

BAL (0.22 cc saturated aqueous soln.) prolongs A-V and I-V conduction times and Q-T intervals at all rates of stimulation (R-R intervals). Despite these deleterious effects, the total mercurhydrin tolerated thereafter amounts to 780 mg of mercury/kg of heart weight, or more than 10 times the usual cardiolethal dose of the mercurial. Times—sec.

phylline, 6 with mercuzanthin, and 40 with mercurhydrin, constitute the basis of the following data. The rabbits weighed 1.5 to 2 kg and the hearts 4 to 6 g. The perfusate volume was 1 liter. Hence, to translate dosages of mg/kg of heart weight into concentrations of mg % of perfusate, one need only

1000  
divide by the factor 20 (i.e. —). Dosages  
5 x 10

on figures per kg of body weight must be multiplied by the factor 200 to get approximate values per kg of heart weight.

**Results 1. Effects of Mercurials.** Salyrgantheophylline and mercuzanthin were usually rapidly (within 10 minutes) fatal to the rabbit hearts at doses of 30 mg of mercury/kg of heart weight, or above. Block of severe degree, A-V nodal or intraventricular (QRS widening of 50 to 150%), generally occurred at a dosage of 7.5 mg of mercury/kg or above. That mercurhydrin was less toxic, as noted by

previous investigators,(10) was emphasized by its cardiolethal dosage of 75 mg of mercury/kg or over, and the occurrence of marked impairment of conduction following doses of 36 mg of mercury/kg or over (Fig. 2, 4, and 6; Table I).

2. *BAL and Mercurhydrin.* BAL in saturated aqueous solution (4.5%), administered in a dosage of 1800 mg/kg of heart weight caused some impairment of conduction and lengthened the Q-T interval. The dosage of mercurhydrin tolerated thereafter, however, increased up to 780 mg of mercury/kg without immediately killing the heart, although further deleterious changes in conduction and refractory period followed (Fig. 1). When conduction impairing doses of mercurhydrin (e.g. 36 mg of mercury/kg) were followed by BAL 1800 mg/kg, there resulted a marked re-

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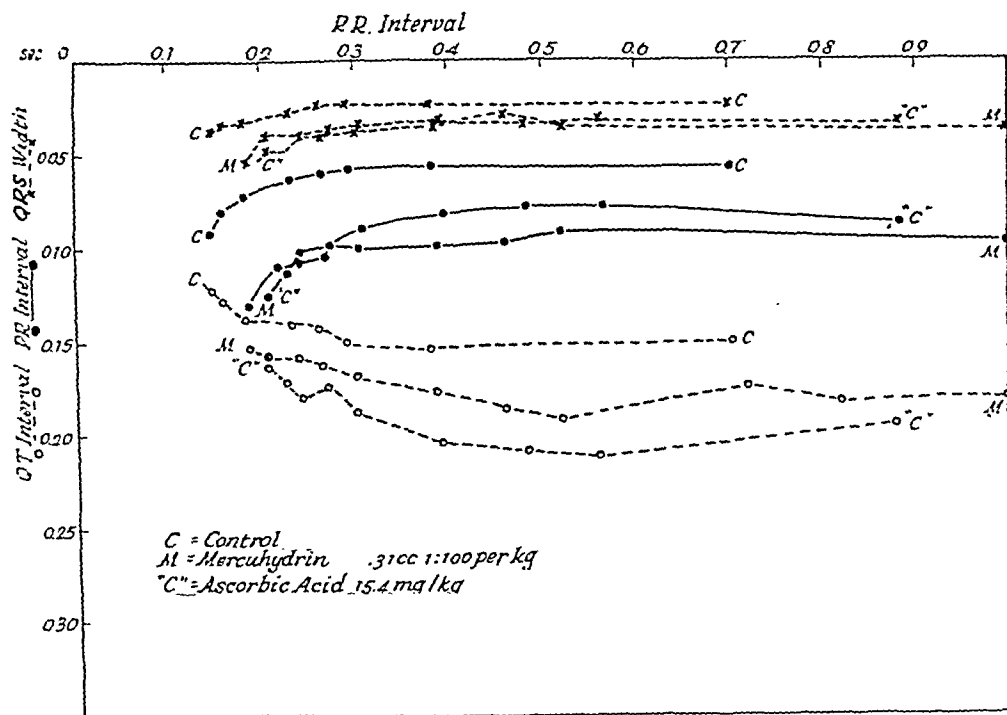


FIG. 4.

The usual effects of a blocking dose of mercurhydrin (36 mg of mercury/kg of heart weight) are reversed as regards A-V and I-V conduction only slightly, and at lower rates of stimulation, by ascorbic acid in this preparation; the electrical systole is further prolonged.

the Q-T interval and the absolute refractory period of conductivity.(11)

*Comment.* Since the lethal effects of intravenous mercurial diuretics result primarily from cardiac toxicity,(2) it is pertinent to compare the doses of mercurials fatal for the isolated rabbit hearts with the known M.L.D.'s of these drugs in intact animals. In terms of mercury content, mercurhydrin was fatal in dogs in a dose of 45-61 mg/kg of body weight,(1) and salyrgan-theophylline in cats at 35 mg/kg.(2) Our corresponding figures for the two drugs are 75 mg/kg and 30 mg/kg of heart weight respectively. These doses of mercurials produce rapid cessation of cardiac activity as compared with the slower cumulative effects of the M.L.D.'s as defined for intact animals. Although our results are not quantitative, it appears that BAL, ascorbic acid and thiamin offered, in that order, significant protection to the heart against mercurial toxic and lethal effects. *The maximum tolerated (sublethal) doses of mercurhydrin*

*following BAL, ascorbic acid, and thiamin, were 10.0, 7.6, and 2.6 times the previously noted M.L.D. of the mercurial alone in our preparation of the perfused isolated rabbit heart, respectively.* Each of them, likewise, showed definite reversal of mercury-induced conduction defects and, usually, electrical systole prolongation.

Further studies indicate that mercaptomerin (the disodium salt of N (γ-carboxymethyl-mercaptopmercuri-β-methoxy)propyl camphoramic acid, thiomerin-Campbell Products) is less than 1/200 as cardiotoxic as mercurhydrin. Slight cardioprotective effects also result from the exhibition, before or after thiomerin, of BAL, but not of thiamin or ascorbic acid.(14) The mechanism of BAL detoxication of mercury has been adequately discussed elsewhere.(4) We can offer no explanation of the actions of thiamin and ascorbic acid, other than possible

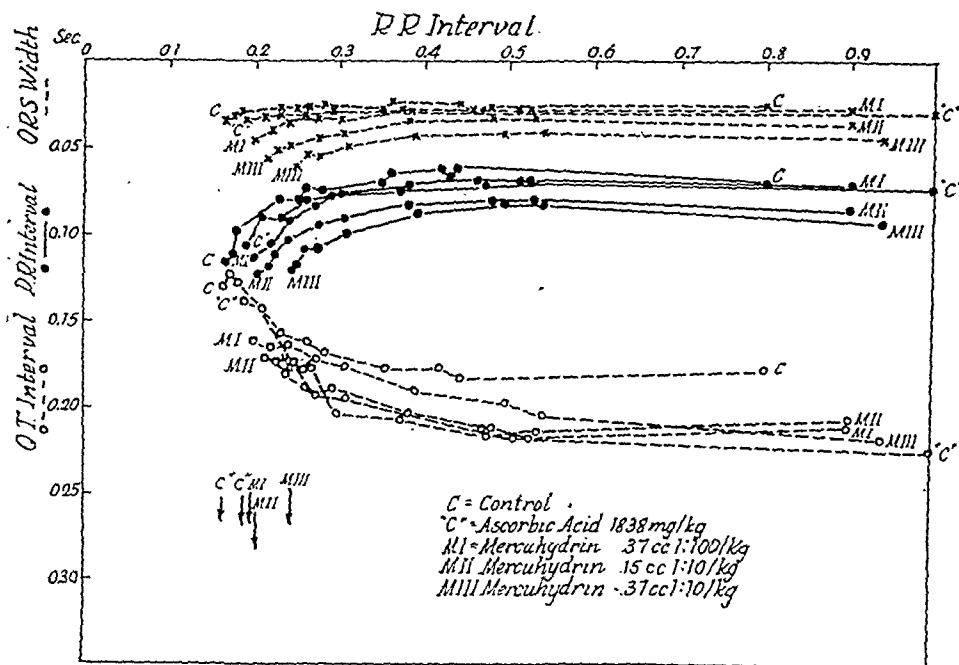


FIG. 3.

Ascorbic acid has practically no effect on this heart preparation except for prolonging the Q-T interval and slightly decreasing the blocking stimulus rate (see arrows). 570 mg of mercury/kg of body weight, or more than 7 times the usual cardiolethal dose, is tolerated thereafter with few further deleterious effects on the measured intervals.

min administered in above doses after blocking doses of mercurhydrin caused partial reversal of the prolonged P-R and QRS intervals, while prolonging the Q-T intervals further (Fig. 6). This action was similar to but less marked than that of BAL and ascorbic acid under similar conditions (supra).

5. *Other drugs and mercurials.* Theophylline ethylene diamine in doses of 3600 mg/kg apparently increased the tolerance of the isolated rabbit heart to mercurhydrin (e.g. 120 mg of mercury/kg with non-lethal A-V dissociation, Table I), while itself causing some block and particularly prolongation of the electrical systole.(11) The protective effects of theophylline have been previously reported,(2) associated, perhaps, with the increased absorptive and diuretic results of its combinations with mercurials,(2,12) as well as coronary dilation since pitressin had the opposite effect (Table I). Epinephrine, 6 mg/kg, partially reversed the effects of blocking doses (36 mg of mer-

cury/kg) of mercurials on the P-R, QRS and Q-T intervals, without causing ventricular fibrillation.(11) DeGraff and Lehman(2) had previously reported "improvement" in hearts poisoned by mercurials following ephedrine. Since magnesium sulphate has been reported to have detoxifying value against mercurial diuretics,(13) doses up to 45 to 120 g/kg were employed before and after mercurhydrin with failure to demonstrate any protective or beneficial effects. Marked A-V (not I-V) block and prolongation of the Q-T interval were produced following  $MgSO_4$  both before and after mercurhydrin, and the cardiolethal dose of the latter was still 75 to 125 mg of mercury/kg after  $MgSO_4$ . Niacin likewise failed to increase the tolerance of the isolated rabbit heart to mercurials, and in doses much larger than the corresponding therapeutic ones in man, e.g. up to 9000 mg/kg, caused slight impairment of conduction (A-V, rather than I-V), and prolongation of

12. Ruskin, A., and Herrmann, G. R. *J. Lab. and Clin. Med.*, 1944, v29, 486.

13. Pines, I., Lanabria, A., and Arriens, R. T. H., *Brit. Heart J.*, 1944, v6, 197.

TABLE I.

Representative Doses of Mercurhydrin and Other Mercurials in mg/kg Heart Weight Producing Marked Conduction Delays (50% to 150% in QRS Width at 150 Beats/min.) and Rapid (Within 10 Minutes) Asystole in Isolated Perfused Rabbit Hearts.

Conduction delays mg/kg		Rapid asystole mg/kg
Salyrgan-theophylline		
7.9		29.6
10.8		36.2
11.8		44.1
Mercuzanthin		
7.4		30.8
12.6		46.7
44.2 (after thiamin)		105.4 (after thiamin)
Mercurhydrin		
17.8 (after pitressin)		75.8
29.8		88.2
30.4		95.6
38.8		112.7
40.0		116.2 (after MgSO <sub>4</sub> )
40.7		118.9
42.1		124.6
44.1		144.4
48.6		148.5
48.8		149.4
53.9		169.4 (after thiamin)
56.1		195.2 ( " " )
62.3		356.7 (after ascorbic acid)
66.8		418.8 ( " " " )
120.4 (after aminophylline)		570.6 ( " " " )
242.6 (after BAL)		696.6 ( " BAL)
		780.5 ( " " )

potentiation of the cellular enzyme systems incompletely destroyed by mercury. Biochemical studies, utilizing the Warburg technique, now being conducted in our neuro-chemistry laboratory, may substantiate this hypothesis.(15) Other substances concerned with cellular oxidation, such as niacin and riboflavin, are without effect on mercurial toxicity.(11) The favorable effects of theophylline ethylene diamine and epinephrine confirm previous similar observations. Magnesium salts, while apparently stimulating the contractility of the preparation,(11) produce, with mercurhydrin, additional deleterious conduction effects, without changing the cardiolethal dosage of the mercurial compound.

**Summary.** Mercurhydrin uniformly caused conduction delays in the A-V node and ventricles of the isolated rabbit heart. Electrical systole (Q-T interval) was likewise prolonged,

15. Hussey, M. S., and Nowinski, W., in progress.

and failure of A-V conduction (dropped beats) produced at lower than usual rates of stimulation—all at doses above 36 mg of mercury/kg of heart weight. Rapid cardiac asystole resulted from doses above 75 mg of mercury/kg of heart weight. Preliminary BAL, ascorbic acid, and thiamin, in descending order of efficacy, raised the blocking and cardiolethal dosages of mercurhydrin, and the same drugs administered following the mercurial compound partially reversed the cardiac effects measured. Theophylline ethylene diamine and epinephrine exhibited lesser cardio-protective activity. Magnesium sulphate and niacin had none.

The authors are indebted to Mrs. J. E. Johnson of the Department of Biological Chemistry for pH determinations of the perfusion fluids and to Dr. W. J. Wingo, formerly of the Department of Biological Chemistry, for checking the degree of solubility of BAL in aqueous solutions.

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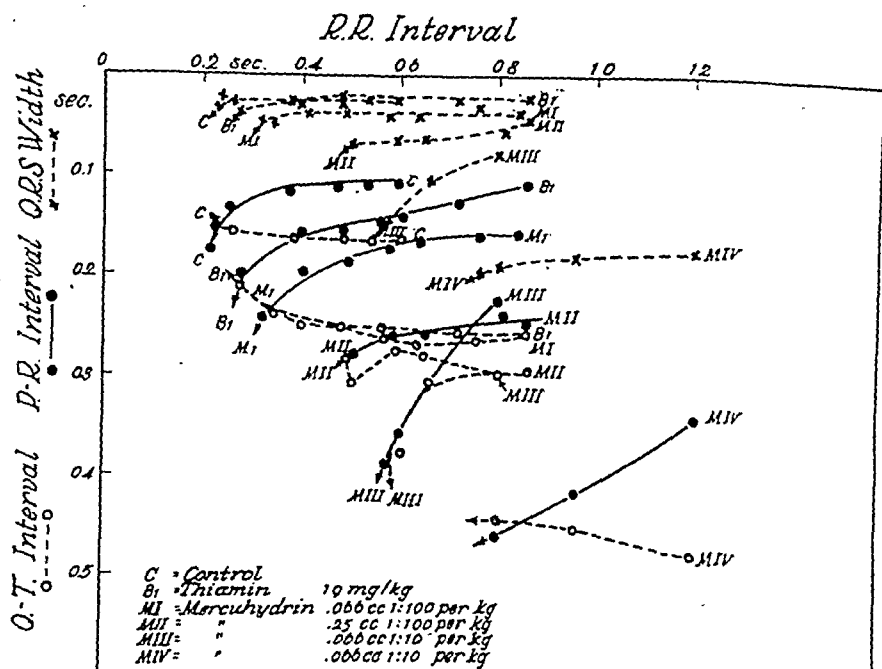


FIG. 5.

The large dose of thiamin has increased A-V conduction time and the Q-T intervals at all rates of stimulation in this heart. Nevertheless, mercurhydrin is tolerated, thereafter, though with markedly deleterious effects, up to 195 mg of mercury/kg of heart weight, or almost 2 times the usual cardiolethal dose of mercurhydrin alone.

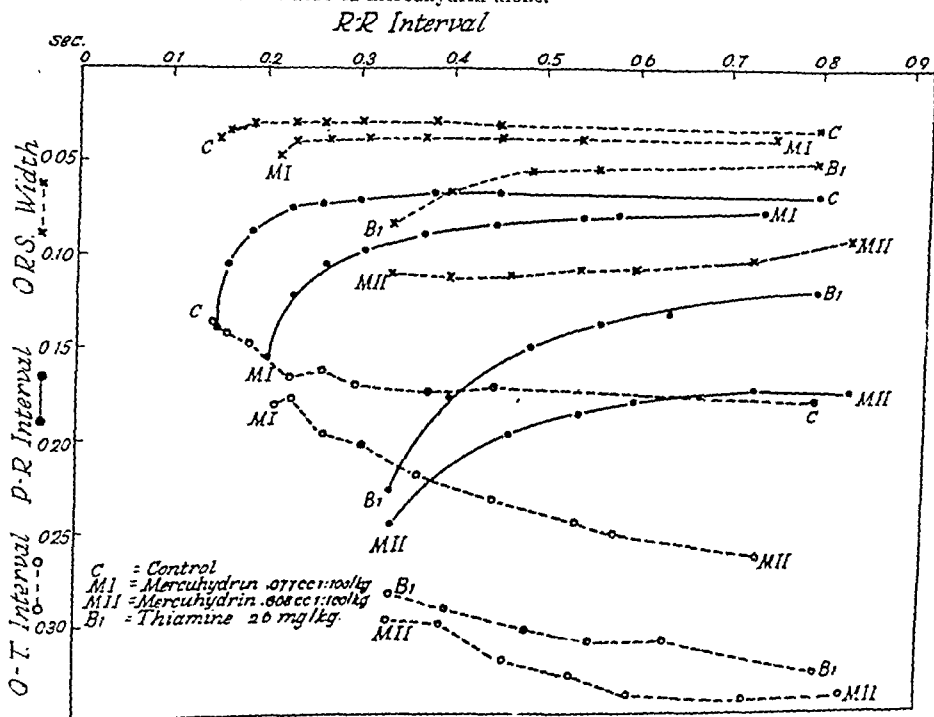


FIG. 6.

A total of 45 mg of mercury/kg of body weight administered in two successive doses of mercurhydrin has caused marked prolongation of A-V and I-V conduction and electrical systole in this preparation. Thiamin then effected marked shortening of conduction times and a slight shortening of Q-T intervals without changing the blocking stimulus rate.

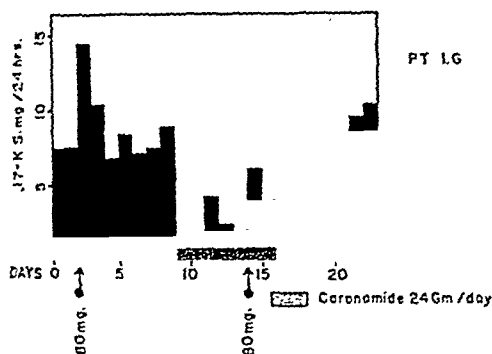


FIG. 2.

The modification of the expected sharp rise in 17-ketosteroid excretion following the intramuscular injection of testosterone by concomitant administration of caronamide.

became stabilized again, he was given 24 g of caronamide in divided dosage per 24 hours for 7 days. On the fifth day of this period he was given another injection of 80 mg of testosterone. The excretion of 17-ketosteroids following the injection of testosterone while the patient was receiving caronamide was distinctly less than that following the previous injection when the subject was not receiving the drug. However, when caronamide was discontinued 48 hours following the second injection, there was an immediate increase in steroid excretion.

**Discussion.** The data in Exp. 1 would seem to indicate that in sufficient dosage, caronamide will inhibit the excretion of endogenously manufactured 17-ketosteroids. The apparent absence of any "rebound" after the discontinuance and elimination of the drug might suggest increased degradation of these substances to non-17-ketosteroid derivatives when their natural avenue of excretion is

blocked, or their alternate elimination by some other excretory route.

In reference to Exp. 2, it should be stated that in data not herein presented, we have evidence that caronamide must be given in sufficient dosage to effectively block renal tubular function if the excretion of administered testosterone is to be modified effectively. This has been shown true for penicillin also. (7) It is of some interest that the immediate, expected sharp rise in 17-ketosteroids following the injection of testosterone during the drug period was modified considerably, but that such an increase did occur following the cessation of the drug 48 hours later. This might suggest that the degradation products of administered testosterone are not further degraded rapidly when their natural pathway of excretion is blocked, and that renal tubular excretion may possibly represent their selective excretory route. It would also seem logical to assume from Exp. 1 and 2 that 17-ketosteroid excretion involves a renal tubular mechanism very similar to that for para-aminohippurate, phenolsulphonphthalein and penicillin. Further data must be obtained to substantiate these theories.

**Conclusions.** Caronamide (4'-carboxyphenylmethanesulphonanilide) has been found to inhibit the excretion of 17-ketosteroids in man. In effective dosage it will likewise apparently modify the expected rise in 17-ketosteroids following the intramuscular injection of testosterone.

7. Boger, W. P., Kay, C. F., Eisman, S. H., and Yeoman, E. E., *Am. J. M. Sc.*, 1947, v214, 493.

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# Inhibition of the Urinary Excretion of 17-Ketosteroids by Caronamide. (17505)

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(Introduced by David K. Miller)

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The renal excretion of penicillin has been effectively inhibited by the concomitant administration of the drug, Caronamide (4'-Carboxyphenylmethanesulphonanilide)(1). Caronamide does so by inhibiting the transport mechanism responsible for the tubular excretion of penicillin.(2) It has been demonstrated that caronamide inhibits also the excretion of paraaminohippurate and phenol-sulphonphthalein but has no effect on the glomerular filtration rate, the Tm of glucose or arginine, or the clearance of urea or sulfonamides.(3) It was thought to be of interest to study the effect of this compound on the urinary excretion of 17-ketosteroids in man.

**Material and methods.** The subjects studied were hospitalized ambulatory patients receiving the routine hospital diet. At no time during the study was any patient under any unusual "stress." Twenty-four-hour urine collections were checked for accuracy by creatinine determinations. Determination of creatinine was by the method of Folin(4) and of 17-ketosteroids by the method of Fraser *et al.*(5)

**Exp. 1.** After a control period of 2-3 days, during which 17-ketosteroid levels were determined, 1 female and 3 male patients received 24 g of caronamide\* in divided doses

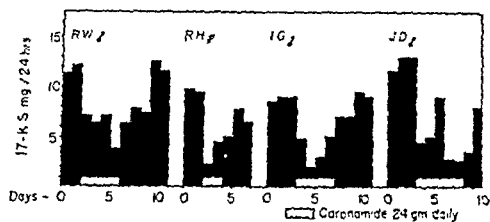


FIG. 1.

The inhibition of 17-ketosteroid excretion by the administration of caronamide. The sudden rise in steroid excretion in the case of Patient J.D. on day 6 of the study was apparently due to the patient's forgetting to take all his drug on this day, which he admitted on questioning.

per 24 hours. The female received the drug only 2 days; the males for longer periods. Each male had some degree of gastric distress toward the end of the drug period. Each subject had a sharp drop in the 17-ketosteroid level in the urine during the administration of drug, followed by a return to normal after a slight lag period (Fig. 1). This "lag" is probably due to continued elevated levels of caronamide due to accumulation of the drug, but since no blood caronamide levels were performed it is impossible to prove this point.

**Exp. 2.** The injection of testosterone into the human is accompanied by an immediate increased excretion of 17-ketosteroids.(6) An experiment was devised to attempt to determine whether this increased excretion could be modified by caronamide administration.

Patient I.G., a eunuchoid male who had been studied in Exp. 1, was given a single injection of 80 mg of crystalline testosterone in aqueous suspension<sup>†</sup> after a 48 hour control period (Fig. 2). This was followed by a sharp increase in 17-ketosteroid output. Five days later, after the patient's steroid excretion

1. Beyer, K. H., Miller, A. K., Russo, H. F., Patch, E. A., and Verwey, W. F., *Am. J. Physiol.*, 1947, v149, 355.

2. Beyer, K. H., *Science*, 1947, v105, 94.

3. Beyer, K. H., Russo, H. F., Patch, E. A., Tillson, E. K., and Shaner, G., *J. Pharm. and Exp. Therap.*, 1947, v91, 272.

4. Folin, O., *J. Biol. Chem.*, 1914, v17, 469.

\* Caronamide (Statacin) was kindly supplied by Dr. W. P. Boger, Sharpe & Dohme Co., Glenolden, N.J.

5. Fraser, R. W., Forbes, A. P., Albright, F., Sulkowitch, H., and Reifstein, E. C., Jr., *J. Clin. Endocrinol.*, 1941, v1, 234.

6. Dorfman, R. I., and Hamilton, J. B., *J. Clin. Invest.*, 1939, v18, 67.

<sup>†</sup> Kindly supplied by Dr. George Hazel, Abbott Laboratories, North Chicago, Illinois.

exo- or endoerythrocytic infection during the 35-day observation period.

The second experiment included 45 week-old New Hampshire Red chicks, divided into 3 groups of 15 birds each. Each chick received 0.01 mosquito equivalent of sporozoites injected into the pectoral region. The treatment schedule, dosage of aureomycin for each of the 3 groups (A, B and C) and blood smear examination was exactly the same as described for the preceding experiment. In the control series, all of the chicks had detectable parasitemia 8 to 10 days after inoculation. Only 4 of the 15 chicks in group A and 2 of the 15 chicks in group B developed malaria. Whereas 11 of the 15 control chicks died of an overwhelming tissue infection, only 1 of the 30 treated chicks died during the observation period of 35 days.

*Action against late exoerythrocytic forms.* This experiment included 15 New Hampshire Red chicks one week old at the time of inoculation with 0.01 mosquito equivalent of sporozoites of *P. gallinaceum*. Five chicks were given aureomycin orally at 100 mg/kg twice daily for 7 days beginning at the time parasites were first detected in the blood, which in this test was 9 or 10 days after inoculation. The other 10 birds were kept as controls. On

the 13th day after infection 9 of the 10 control chicks were dead and autopsies of their brains showed an overwhelming exoerythrocytic infection. (The remaining control chick survived until sacrificed 31 days after infection; no exoerythrocytic parasites were demonstrable). One of the 5 aureomycin-treated chicks died of an exoerythrocytic infection after 2 doses of the drug. The other 4 treated chicks survived until sacrificed 31 days after inoculation and no exoerythrocytic parasites were demonstrable at autopsy.

Although radical cures were not obtained, since erythrocytic parasites were demonstrable beginning 4 to 8 days after cessation of treatment, aureomycin was able to prevent death from exoerythrocytic forms, a property previously exhibited only by certain 8-aminoquinolines (pamaquine and pentaquine), chlorguanide (paludrine) and sulfadiazine.(3)

*Summary.* Aureomycin acted as a causal prophylactic and was therapeutically active against erythrocytic parasites and late exoerythrocytic forms of *P. gallinaceum* in the chick.

3. Greenberg, J., Trembley, H. L., and Coatney, G. R., *Am. J. Hyg.*, in press.

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### Aureomycin in Experimental Chesson Strain Vivax Malaria. (17507)

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Aureomycin has been given a limited trial against sporozoite-induced Chesson strain vivax malaria in white male volunteers, and the results, although still incomplete, warrant a brief progress report. The procedures used

1. Coatney, G. R., Cooper, W. C., and Ruhe, D. S., *Am. J. Hyg.*, 1948, v47, 113.

2. Coatney, G. R., Greenberg, J., Cooper, W. C., and Trembley, H. L., *Proc. Soc. Exp. Biol. and Med.*, 1949, v72, 586.

\* Aureomycin was furnished by courtesy of Doctor Stanton M. Hardy, Lederle Laboratories Division, American Cyanamid Company.

were those employed throughout our program of screening speculative antimalarial drugs.(1) Very high dosages of aureomycin were given, approaching the range found by Coatney *et al.*(2) to be effective against *Plasmodium gallinaceum* in the chick.

*Protective action.* Two volunteers were each given 8 g of aureomycin\* per day (1 g every 3 hours) on the day of bites by 10 heavily infected *Anopheles quadrimaculatus* mosquitoes, and for 6 days thereafter. They did not develop patent malaria until 30 and 31 days after exposure, whereas 4 controls

# Antimalarial Activity of Aureomycin Against *Plasmodium gallinaceum* in the Chick. (17506)

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During the routine testing of several antibiotics for antimalarial activity, aureomycin was found active against *Plasmodium gallinaceum* in the chick. This is the first demonstration that aureomycin has activity against any malarial parasite.

**Action against erythrocytic parasites (A-1 test).**(1) This series of experiments was designed to test for action against erythrocytic parasites. Week-old New Hampshire Red chicks were inoculated intravenously with  $16 \times 10^6$  parasitized erythrocytes. Treatment was begun 4 to 5 hours before inoculation and continued twice daily for 4 days. On the morning of the 4th day (day of inoculation is day 0) blood smears were examined and estimations made of the number of parasitized erythrocytes per  $10^4$  erythrocytes. In the first test aureomycin,\* dissolved in water, was

administered by injection into the pectoral muscles at a dose of 80 mg/kg. This regimen resulted in a significant reduction in parasitemia (Experiment 1, Table I). The chicks gained weight normally and there was no evidence of tissue damage at the site of inoculation. In Experiments 2, 3, 4 and 5 the aureomycin was administered orally. As shown in Table I, 75 mg/kg in all instances resulted in a pronounced reduction in parasitemia. Per unit weight the drug is thus approximately one-fourth as effective as quinine.(1)

**Action against early exoerythrocytic parasites (A-2 test).**(1) The first test for causal prophylaxis included 30 chicks, New Hampshire Reds, one week old, divided into 3 groups of 10 birds each. Each chick received one mosquito equivalent of sporozoites injected into the pectoral region.(2) Treatment was begun 4 to 5 hours before inoculation and continued twice daily for 4 days. Group A received aureomycin orally at a dosage of 75 mg/kg twice daily, group B received 150 mg/kg twice daily, and group C served as the control. Blood smears were examined daily from the 7th through the 20th day, then every other day through the 35th day after inoculation. All control birds had detectable erythrocytic parasites between the 7th and 9th day after inoculation. Seven of the 10 birds in group A developed infections, the first on the 9th and the last on the 15th day after inoculation; in 50% of the group patency was delayed for 6 days beyond that of the controls. In group B, only 5 of the 10 birds developed patent infections, the first on the 11th day and the last on the 22nd day after inoculation. Whereas 9 of the 10 control birds died of an overwhelming exoerythrocytic infection (days 9 to 15), only 5 of the 20 treated chicks died as a result of either

TABLE I.  
Activity of Aureomycin Against Blood-Induced *Plasmodium gallinaceum* in the Chick.

Exp. No.	Dosage of aureomycin mg/kg b.i.d. for 4 days	Parasitized r.b.c. in treated birds as compared with controls (%)
1	80*	12†
2	80	39†
3	75	4†
	150	6†
4	37.5	80
	75	33†
5	18	69
	37.5	63
	75	44†
	150	16†

\* Intramuscular; all other dosages oral.

† The probability of this reduction in parasitemia occurring by chance is less than one in one hundred.

1. Coatney, G. R., and Sebrell, W. H., in Wiselogle, F. Y., *A Survey of Antimalarial Drugs, 1941-1945*, J. W. Edwards, Ann Arbor, Mich., 1946.

\* Aureomycin was kindly supplied through the courtesy of Doctor J. M. Rueggsegger of the Lederle Laboratories Division, American Cyanamid Company.

2. Coatney, G. R., Cooper, W. C., and Trembley, H. L., *Am. J. Hyg.*, 1945, v42, 323.

# Influence of Time of Ingestion of Essential Amino Acids upon Maintenance of Nitrogen Balance.\* (17508)

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Accumulating evidence supports the assumption that all of the essential amino acids must be available in the diet simultaneously for growth or weight gain.(1-4) Previous experiments from this laboratory(3) demonstrated that protein-depleted rats did not gain weight when 2 incomplete essential amino acid mixtures (which when mixed constituted a complete mixture) were offered in rotation even an hour apart. However, because of the abrupt loss of appetite resulting from feeding rations deficient in one or more essential amino acids,(5) it was difficult to ascertain how much of the recorded loss of body weight was due to inanition and how much to failure of supplementation of one deficient mixture by the other. Similarly poor food consumption may have influenced the results of others who have reported similar experiments in the weanling rat. (1,2) In view of the theoretical and practical importance of this problem to man, whose appetite apparently is not reduced so greatly by the absence of a complete mixture of the essential amino acids, the present experiment was performed. It was planned so that the alternate ingestion of 2

incomplete essential amino acid mixtures, which together contained all 9 amino acids essential for growth and maintenance of the rat,(5-8) could be accomplished while all food constituents were being supplied at an adequate level.

*Plan.* Two groups of 6 adult male Sprague-Dawley rats of comparable weight and age were force fed all of their food at 12 hour intervals for 14 days. For 7 days the first group received alternately ration 5A containing 5 of the 9 essential amino acids and ration 4A containing the other 4 essential amino acids. During this period the second group received at each feeding a complete ration

TABLE I.  
Amino Acid Composition of Mixtures Force Fed Normal Rats.

Amino acids		Rations		
		MAA g per 100 g of dry ration	4A	5A
DL	Isoleucine	.66	1.32	—
DL (+)	Lysine HCl	.16	.32	—
DL	Phenylalanine	.15	.31	—
DL	Tryptophane	.06	.11	—
L (+)	Histidine HCl	.08	—	.15
L (+)	Leucine	.21	—	.41
DL	Methionine	.19	—	.37
DL	Threonine	.27	—	.55
DL	Valine	.51	—	1.03
Total essential		2.29	2.06	2.51
DL	Alanine	.58	.58	.58
L (+)	Arginine HCl	.51	.51	.51
DL	Aspartic acid	.65	.65	.65
L (—)	Cystine	.04	.04	.04
L (+)	Glutamic acid	2.44	2.44	2.44
	Glycine	.05	.05	.05
L (—)	Tyrosine	.66	.66	.66
Total non-essential		4.93	4.93	4.93

\* The research which this paper reports was undertaken in cooperation with the Navy Department Office of Naval Research. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department. The work has been aided, also, by the National Livestock and Meat Board, and by the Douglas Smith Foundation for Medical Research of the University of Chicago.

1. Berg, C. P., and Rose, W. C., *J. Biol. Chem.*, 1929, v82, 479.

2. Geiger, E., *J. Nutrition*, 1947, v34, 97.

3. Cannon, P. R., Steffee, C. H., Rowley, D. A., and Stepto, R. C., *Fed. Proc.*, 1947, v6, 390.

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5. Frazier, L. E., Wissler, R. W., Steffee, C. H., Woolridge, R. L., and Cannon, P. R., *J. Nutrition*, 1947, v33, 63.

6. Rose, W. C., *Phys. Rev.*, 1938, v18, 109.

7. Benditt, E. P., Humphreys, E. M., Straube, R. L., Wissler, R. W., and Steffee, C. H., *J. Nutrition*, 1947, v33, 85.

8. Wissler, R. W., Steffee, C. H., Frazier, L. E., Woolridge, R. L., and Benditt, E. P., *J. Nutrition*, 1948, v36, 245.

TABLE I.  
Effect of Aureomycin on 4 Primary Attacks of Chesson Strain Vivax Malaria in Volunteers Bitten by 10 Infected Mosquitoes on 30 August, 1949.

Date, Sept.	Volunteer No. 248				252				253				254			
	Parasites, 5000 WBC	Peak fever, °F	Aureo- mycin (g)	Aureo- mycin (g)	Parasites, 5000 WBC	Peak fever, °F	Aureo- mycin (g)	Aureo- mycin (g)	Parasites, 5000 WBC	Peak fever, °F	Aureo- mycin (g)	Aureo- mycin (g)	Parasites, 5000 WBC	Peak fever, °F	Aureo- mycin (g)	Aureo- mycin (g)
11	<10	—	—	—	0	—	—	—	<10	—	—	—	0	100.2	—	—
12	<10	102.4	—	—	<10	—	—	—	0	104.0	—	—	0	105.2	—	—
13	200	99.4	—	—	100	98.4	—	—	230	105.0	—	—	190	100.6	—	—
14	140	104.2	—	—	130	103.6	—	—	190	105.4	—	—	140	105.2	—	—
15	3010	99.2	8.0	8.0	2310	99.2	8.0	8.0	4420	105.0	4	4	940	102.8	4	4
16	1890	104.0	7.3	8.0	780	104.4	8.0	8.0	2350	105.6	4	4	810	106.2	4	4
17	5430	99.6	6.6	6.6	6820	99.6	6.6	6.6	10,120	103.0	4	4	2380	103.2	4	4
18	4480	103.2	0.3	2.3	3350	104.6	2.3	2.3	4100	105.4	4	4	700	106.4	4	4
19	400	99.2	1.5*	1.5*	890	98.8	1.5*	1.5*	50	99.2	1.5*	1.5*	40	99.6	1.5*	1.5*

\* Aureomycin was discontinued and quinine therapy instituted after the blood smears of September 19th had been made but before their examination.

bitten by the same mosquitoes had patent infections beginning 12 to 14 days after exposure. In 92 similarly infected controls of previous tests positive blood smears appeared 10 to 16 days after exposure (mean  $12.0 \pm 0.15$ ). The delay in the aureomycin-treated subjects is interpreted as indicative of antiparasitodal action although complete prophylaxis was not attained.

*Therapeutic action.* The 4 controls from the foregoing experiment were treated with aureomycin. The original plan was to give 2 patients 8 g per day for 7 days and 2 patients 4 g per day for 14 days. The higher dosage caused nausea and vomiting in patients with active malaria, so that at times administration by vein was necessary. As shown in Table I, parasite densities in the blood increased and fever continued unabated until the 5th to 6th day of treatment, when the parasite counts in all subjects dropped precipitously. Unfortunately, because of the apparent lack of response, quinine sulfate therapy was begun on September 19, after the drop in parasite counts had occurred, but before the slides had been examined. This prevents knowledge of how soon aureomycin alone might have resulted in negative blood smears. The point is academic, however, because the action against erythrocytic parasites, although significant in primary attacks, is far too slow for practical usefulness. Relapses occurred in all of the 4 subjects within 10 to 14 days after the completion of quinine therapy (2 g per day for 7 days). Aureomycin, therefore, does not eradicate the fixed-tissue stage of *P. vivax*.

*Summary.* Aureomycin, although not a causative prophylactic against the Chesson strain of *P. vivax*, delayed the appearance of malaria in volunteers for at least 2 weeks as compared with controls. When given therapeutically in high dosage, the antibiotic had a delayed action against erythrocytic parasites, too slow to be of practical value. Infections were not cured.

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mately 260 mg for the 7 day period. On the other hand the rats receiving the complete ration each feeding almost maintained their weights and showed slightly positive nitrogen balances. This latter finding confirms the conclusion that the MAA ration is barely adequate to maintain nitrogen balance and weight when fed at the calorie level of 1200/M<sup>2</sup>. The results were essentially similar in each period. Thus the two sets of data gained by interchanging the diets after 7 days act as mutual checks and minimize the influence of the factors of time and animal variation. The fecal nitrogen values were not appreciably altered by the divided feeding of the essential amino acids. Furthermore, the total nitrogen ingested proved to be quite similar in the 2 groups during the 2 periods varying from 944 to 998 mg per rat for each 7 days. Therefore, the increased urinary nitrogen excreted by the rats receiving the essential amino acids in two feedings of incomplete mixtures at 12 hour intervals accounted almost entirely for the negative nitrogen balance which these animals exhibited. It is interesting to note that the difference between the average 7 day urinary nitrogens of the rats receiving the incomplete rations in rotation and those receiving the complete mixture each feeding was 359 mg. This is slightly greater than the total essential amino nitrogen of 316 mg/week which each rat received.

*Discussion.* These results suggest that the normal adult rat is unable to store even temporarily an incomplete mixture of amino acids for later use. Nor does there appear to be a means of drawing upon the abundant labile protein reserves(11) for a certain specific

amino acid or certain amino acids which are absent from the diet. These data, therefore, confirm those reported for the protein-depleted rat(3) and suggest the rat's loss of appetite was probably a good indication of a lack of supplementation of the one deficient ration by the other. The only other nitrogen balance determinations done under conditions of delayed supplementation of which we are aware are those of Elman(12) who gave dogs tryptophane-deficient hydrolysates intravenously followed 6 hr later by tryptophane intravenously. Under these circumstances he noted a negative nitrogen balance. His experiments were not influenced by the speed of intestinal absorption or any problem of differential absorption of amino acids. Therefore it is of interest that the same type of result is obtained by oral force feeding in the rat if the stomach is allowed to empty before the supplementary ration is given. This inability of the normal adult animal to make up for an essential amino acid deficient even for a short time makes it even more important to avoid oral or parenteral feedings made up principally of incomplete proteins or amino acids especially during disease or convalescence. Further studies are needed to determine the source of the excess urinary nitrogen which is excreted under these circumstances.

*Summary.* The normal adult rat is unable to derive adequate nutritional value from an incomplete essential amino acid mixture which is supplemented 12 hours later by the missing amino acids even when attention is paid to strict equalization of the intakes of all food constituents.

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12. Elman, R., *Proc. Soc. Exp. Biol. and Med.*, 1939, v40, 484.

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11. Madden, S. C., and Whipple, J. H., *Physiol. Rev.*, 1940, v20, 194.

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TABLE II.  
Nitrogen Balance and Weight Changes in Rats Force Fed Incomplete Amino Acid Rations Alternately.

Animal No.	Wt, g	1st 7 days			2nd 7 days		
		Diet	Wt gain or loss, g	N bal., mg	Diet	Wt gain or loss, g	N bal., mg
1	325	4A,	-13	-303	MAA	-1	+ 37
2	328	5A	-13	-214	every	-2	+ 83
3	306	alternated	-7	-328	12	-1	+ 15
4	313	every	-15	-226	hr	-2	+ 85
5	305	12	-9	-264		+10	+ 65
6	322	hr	-15	-276		+ 5	+ 91
Avg	317		-12	-269		+ 2	+ 63
7	332	MAA	-8	+ 33	4A,	-10	-285
8	319	every	-2	+ 50	5A	-12	-225
9	322		-2	+ 7	alternated	-13	-316
10	333	12	± 0	+ 41	every	-17	-241
11	317	hr	-2	+ 9	12	-12	-291
12	308	.	-1	+ 71	hr	-13	-301
Avg	322		-3	+ 35		-13	-262

(MAA) containing the 9 essential amino acids. The rations were prepared so that all of the rats would receive equal quantities of all amino acids as well as the other food constituents every 24 hours, the only difference being the time of ingestion of the essential amino acids. During the second 7 day period the rations were interchanged so that the first group received the complete mixture and the second group received the alternating incomplete rations. Nitrogen balances and weight changes were measured for each 7 day period.

*Rations, method of feeding and nitrogen balances.* The quantities of amino acids in the 3 rations are shown in Table I. The quantities in the complete ration (MAA) were derived from data recently obtained in this laboratory.(9) They represent the minimum quantities of each essential amino acid necessary just to maintain weight equilibrium when fed at the daily level of approximately 3.6 g of amino acids (1.15 g essential and 2.45 non-essential) per kg of body weight and about 1200 calories per M<sup>2</sup> of surface area. The concentrations of essential amino acids in each of the other rations (5A and 4A) were double those in MAA in order to make the total daily intakes equal. The remainder of each of the

rations in the "dry" form was made up of dextrin 64%, corn oil 4%, ruffex 5%, salt mixture 4%, water 16% and vitamins in quantities previously reported.(5) Each of the "dry" rations was diluted with additional water to make a thin soup which was thoroughly mixed in a Waring Blendor and fed in 13 ml quantities each feeding. This quantity supplied about 3.84 g of amino acids per kg and 1280 calories per M<sup>2</sup> of surface area per 24 hours. The rations were force fed by the method of Shay and Gruenstein.(10) The rats were adapted to this method of alimentation during a preliminary 6 day period by force feeding gradually increasing quantities of the MAA ration to each rat. Nitrogen balances were determined by methods previously described.(8) Fecal and urinary nitrogens were determined separately. Nitrogen intake was estimated by means of Kjeldahl determinations on multiple aliquots of the diluted rations.

*Results.* A brief summary of the experimental results is presented in Table II. The ingestion of the two incomplete amino acid mixtures at 12 hour intervals led to a weight loss of about 2 g per day per rat and an average negative nitrogen balance of approxi-

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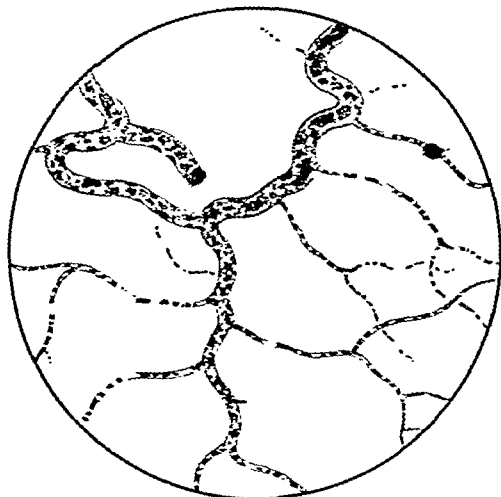
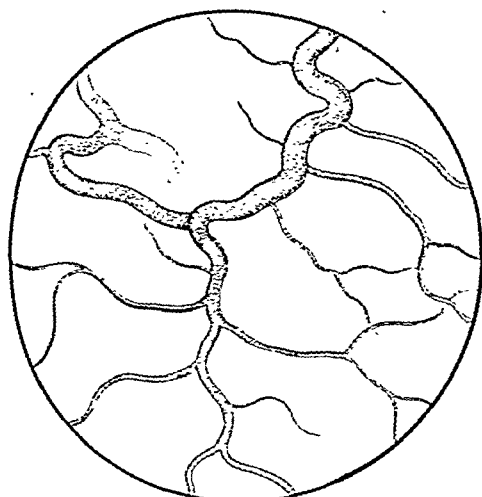


FIG. 1. (Left) Sketch of a normal conjunctival field  $\times 100$  approx.

FIG. 2 (Right) Sketch of the same conjunctival field after cervical sympathetic trunk stimulation or injection of intravenous epinephrin. The smudge, upper right, is a small hemorrhage.

peared. The changes can be repeated several times if one is careful to avoid a hot illuminating light and the conjunctiva is prevented from drying with occasional drops of Ringer's solution. If the stimulation is continued for 60 seconds the sludging effect lasts five to six minutes and some clumping may be seen in the opposite eye.

Local application of 3 to 4 drops of epinephrine (1:10,000-1:50,000) produces a similar effect for a short time. A dilution of 1:1,000 usually produces prolonged stasis and a heavy sludge in most of the superficial vessels. One is immediately led to believe that the temporary hemostatic effect of epinephrin, so often used by surgeons, is not only due to contraction of the vessels, but also due to the creation of the multiple small "sludges" and only in some cases by thrombi in the vessels affected by the drug. Thus we have two mechanisms rather than one in the creation of hemostasis with local epinephrin when applied topically. The release of the corklike masses of "sludge" with dilatation of the vessels as the effect of the drug wears off may be an additional factor in the rather temporary character of the hemostasis produced in this way. A generalized "sludging" effect is produced by intravenous injection of epinephrin. Depending upon the speed of injection and the strength of the solution (1:1,000,000-1:10,000) almost any degree of

blood "sludging" can be produced in a few seconds. Minute doses produce the faintest demonstrable clumping of the red blood cells, but increasing the dosage produces more and larger aggregates of cells until there is complete arrest of blood flow in the peripheral and central circulation with death of the animal. Very occasionally there are changes in capillary wall permeability which permits a few red cells to escape into the tissues. (Fig. 2, upper right). The effect of Nor-adrenalin is similar to epinephrin, but the response is much more transient.

Intravenous injection of novocaine in small doses will often reverse the sludging process, at least for a time, but this varies with the concentration of novocaine and the speed of injection. Further elaboration of the above experiment in various parts of the body and the various diseases now being studied for "sludge" should be interesting and illuminating.

Since fright or anxiety can produce stimulation of the sympathetic system(4) and blood sludge can be produced by sympathetic stimulation and since "blood sludging" has already been observed in psychotic states(2) one can not help calling attention to the possible occurrence of "blood sludge" and its

4. Cannon, Walter B., *Bodily Changes in Fear, Hunger, and Rage*, D. Appleton, 1929.

# Capillary Circulation with Changes in Sympathetic Activity.\* I. Blood Sludge from Sympathetic Stimulation. (17509)

EDMUND P. FOWLER, JR. (Introduced by W. S. Root)

From the Department of Otolaryngology, College of Physicians and Surgeons, Columbia University, New York City.

A study of the effects of sympathetic stimulation on the blood vessels of the conjunctiva of cats was undertaken incidental to some work on the physiological response of the nasal mucous membrane, but the findings seem of such general interest that it has been decided to report them separately. Suffice it to say here, that changes similar to those found in the blood vessels of the conjunctiva have been observed in the frontal sinuses and in the bullae of the middle ear as well as in the blood vessels of the fascia of the scalp. These latter regions are much more difficult to observe than are conjunctivae and there is, of course, much more tissue trauma in the preparation of these areas. This trauma can in itself produce blood sludging.(1)

**Methods.** Normal adult cats were anesthetized with veterinary nembutal, 30 mg per kilo intraperitoneally. The cervical sympathetic trunks were isolated for stimulation with a 60 cycle transformer resistance stimulator using 110 v. A.C. After tracheotomy, the cat's head was held firmly with a Grünfest head holder. The conjunctivae were made more accessible by slitting the external canthi with scissors and retracting the upper lids with traction sutures. The vessels of the eye were observed through a dissecting microscope (Leitz  $\times 60$ - $\times 120$  magnification) using direct illumination from a dissecting lamp with two heat absorbing glass filters. The result is a clear view of venule and capillary circulation (Fig. 1). The arterioles are deeper and harder to see and are therefore not included in the diagram. The intact cervical sympathetic trunk was lifted gently from its bed and was stimulated on the observed side with sufficient current ( $\frac{1}{4}$  to  $\frac{1}{2}$  volt) to produce widening of the pupil.

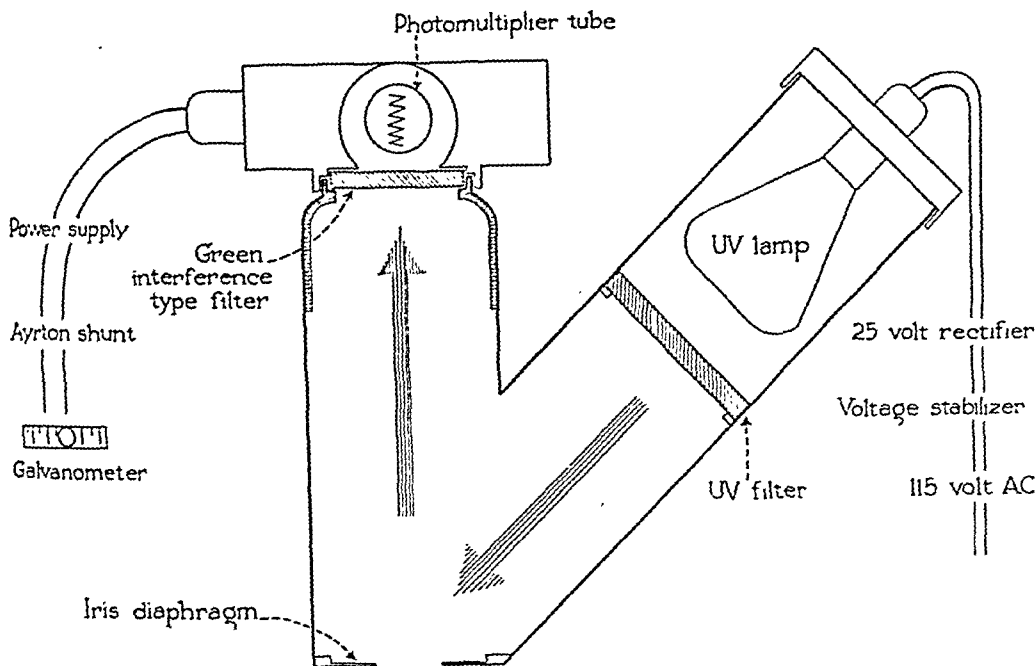
**Observations.** Within a few seconds after cervical sympathetic trunk stimulation the arterioles and many of the venules of the conjunctiva can be seen to decrease in caliber. The red cells in the finer capillaries disappear or become very sparse. Meanwhile the flow in the venules and in at least some of the arterioles is markedly retarded, but in addition there are changes in the blood itself (Fig. 2). The blood cells cluster into clumps which appear identical with those described by Knisely *et al.*(2) as "blood sludge," *i. e.*, adherent aggregates of cells rather than ordinary rouleaux. As mentioned above, in some vessels the blood flow stops, but in a few venules, precapillaries, and capillaries it may reverse. This must be due to the arteriovenous shunts as has been described by Zweifach(3) and others. In somewhat larger vessels the flow continues, as a rule, in the same direction, but at a reduced rate. During stimulation almost all the blood cells in these vessels appear "clumped." In a few fields the clumps of blood cells can be seen squeezing out of a small constricted vessel into a larger vessel like cylinders of tooth paste coming out of a tube. These cylinders of clumped cells do not, as a rule, disintegrate on entering the next larger vessel in which blood flow continues more rapidly. After 15 seconds of stimulation the "sludging" effect increases for several seconds and then slowly disappears after 2 to 3 minutes with a return to normal of the caliber of most of those vessels which had been constricted. Later there seems to be dilatation of some of the vessels. At first, return to normal flow is observed in the larger vessels, then extending to the smaller vessels until all elements of the "sludge" have disap-

\* This work was supported by a grant from the United States Public Health Service.

1. Knisely, Melvin H., Eliot, Theodore S., and Bloch, Edward, *Arch. Surg.*, 1945, v51, 220.

2. Knisely, Melvin H., Eliot, Theodore S., Bloch, Edward, and Warner, Louise, *Science*, 1947, v106, No. 2758.

3. Zweifach, Benjamin W., *Am. J. Anat.*, 1937, v60, 473.



## Fluoro-illuminometer

FIG. 1.

Fluorescence measuring device. (Not drawn to scale).

fluoro-illuminometer (F-I) consists essentially of a metal "Y" tube which serves as a rigid holder for a UV light source, photocell and filters (Fig. 1). The light source is a small 4 watt mercury vapor lamp<sup>†</sup> operating on stabilized rectified current of 20-24 volts. A Corning No. 5874 filter secured beneath the lamp absorbs the visible and transmits the UV radiation between  $\lambda$  310-410  $\mu\mu$ , with a peak transmission at  $\lambda$  365  $\mu\mu$ . The transmitted UV activates the specimen surface to fluorescence. The emitted fluorescence and an undetermined fraction of reflected UV passes normal to the specimen surface through an interference type filter<sup>‡</sup> which functions as a monochromator, transmitting a band of green light which peaks at  $\lambda$  525  $\mu\mu$ . Thus, only the fraction of radiation from the specimen surface corresponding to the peak emission of fluorescein fluorescence reaches the multiplier phototube.<sup>§</sup> The photo current is

read from a sensitive spotlight galvanometer.<sup>||</sup> An Ayrton shunt<sup>¶</sup> included in the circuit permits the utilization of the full galvanometer scale over wide fluctuations of fluorescence intensity without altering phototube sensitivity. The high sensitivity of the photomultiplier tube more than compensates for the rather great transmission loss (60-70%) by the interference filter. Standardization of the apparatus is accomplished by the use of a stable reference material consisting of a 2" x 2" piece of green fluorescent glass<sup>\*\*</sup> seated in a close fitting holder over a thin sheet of polished aluminum foil (Fig. 2). A primary

<sup>§</sup> RCA 1P21.

<sup>||</sup> Rubicon: Sensitivity = .002  $\mu\text{a}/\text{mm}$ ; Resistance = 4800 ohms; CDRX = 110,000 ohms. The phototube and housing, power supply, control unit and galvanometer were purchased as an integrated unit from Farrand Optical Co., Inc., New York City.

<sup>¶</sup> Leeds and Northrup No. 2164, 100,000 ohms.

<sup>\*\*</sup> Canary glass No. 3750, Corning Glass Works, Corning, N.Y.

<sup>†</sup> General Electric, 360 BL RP12.

<sup>‡</sup> Half width =  $15 \pm 5 \mu\mu$ ; Farrand Optical Co., Inc., New York City.

consequences from emotional stimulation of the sympathetic pathways in the many diseases thought to be psychosomatic.

**Summary.** Stimulation of the cervical sympathetic trunk, application of local epinephrin, and injection of epinephrin intravenously has been found to produce, not only the classical picture of decreased circulation from narrowing of the vascular bed, but also what looks like blood "sludge" within the vessels.

It is suggested that this may be of interest to those studying small vessel circulation and "sludge" and may have applications in general surgery, general medicine, and psychosomatics.

The author was assisted in these studies by Drs. Robert D. O'Malley, William A. Jarrett, and Oeta C. Leigh.

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## Quantitative Measurement of Cutaneous Fluorescein Fluorescence as Indicator of the Capillary Circulation.\* (17510)

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When sodium fluorescein is administered intravenously, it rapidly enters the interstitial fluid compartment. Due to its strong green fluorescence, its presence can be readily detected even in low concentrations when the skin, mucosa, or the surface of organs are viewed in suitably filtered long wave UV in a darkened room. Recently the passage of fluorescein into the interstitial fluid spaces was described as a function of capillary permeability.(1,2) However, no conclusive experiments designed to demonstrate this relationship have appeared in the literature. Although the specific factors regulating the passage of the dye through the capillary wall are not known with certainty, it can be clearly demonstrated that the patterns of cutaneous fluorescence, with respect to time and intensity, strongly mirror the capillary status of the region observed. Thus, intracutaneous epinephrine prevents fluorescein fluorescence at the site of injection, whereas histamine, acetylcholine, heat, etc. augment fluorescence.(1-4) Further, the intensity of fluorescence is

quantitatively related to the concentration of the intracutaneously administered drugs as well as the amount of intravenously administered dye.(1,3) On the basis of this cursory relationship between fluorescein fluorescence and vascularity, it seemed worthwhile to refine methods of accurately measuring fluorescence *in vivo* with the intention of providing an objective tool for the quantitative study of the capillary circulation in physiologically intact preparations. Although Lange and Krewer(5) and Crismon and Fuhrman(6) have measured cutaneous fluorescein fluorescence by quantitative methods, experience with components of their apparatus have shown that they lack sufficient sensitivity and control to warrant usefulness as precision detectors of minute changes in fluorescence.

The instrumentation and methods reported herein are a modification of a procedure used for the determination of fluorescence in solids,(7) operating in principle similar to that of the dermo-fluorometer.(5)

**Description of the Apparatus.** The fluorescence measuring device designated as the

\* Supported by a grant in aid from the Graduate School Research Board.

1. Bukantz, S. C., and Dammin, G. J., *Science*, 1948, v107, 224.

2. Lange, K., and Boyd, L. J., *Arch. Int. Med.*, 1944, v74, 175.

3. Schiller, A. A., *Fed. Proc.*, 1949, v8, 139.

4. Schiller, A. A., to be published.

5. Lange, K., and Krewer, S. E., *J. Lab. and Clin. Med.*, 1943, v28, 1946.

6. Crismon, J. M., and Fuhrman, F. A., *J. Clin. Invest.*, 1947, v26, 259.

7. Schiller, A. A., and Cecchini, L. P., Project NM 001 008, Report No. 3, 21 July, 1948, Naval Medical Research Institute, Bethesda, Maryland.

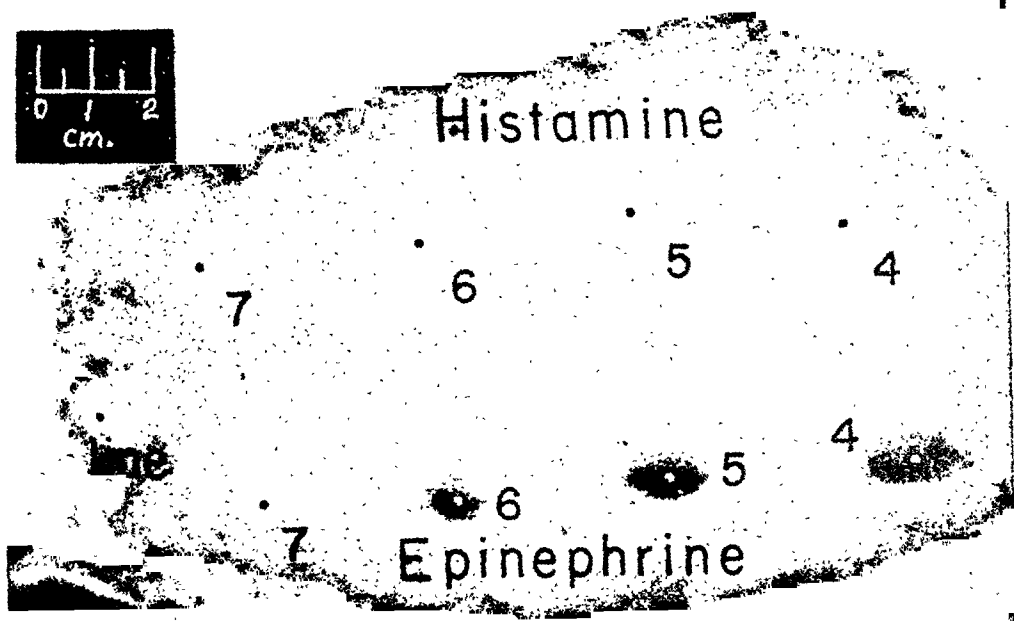


FIG. 3.

UV photograph of whealed rabbit's abdomen 9 min. after i.v. fluorescein (*cf.* Fig 4). The numbers refer to the concentrations of the drugs, *viz.*, 10<sup>-4</sup>, 10<sup>-5</sup>, etc. The black and white dots are artefacts to aid in identifying the center of each wheal.

is found to vary in a regular manner depending on the specific activity and the amount of the drug used as well as the anatomical location of the wheal. (Fig. 3). Time-intensity curves of fluorescein fluorescence are shown (Fig. 4) in which histamine and epinephrine were employed. It will be seen that generally the largest amount of epinephrine and histamine show the least and highest fluorescent intensities respectively, which is consistent with the known activities of these drugs. Also, the several dilutions of either drug produce a readily differentiable response at some point along the time axis which permits quantitative inter-comparison. The opaque colloidal dye, trypan blue, which is reputed to be an indicator of capillary permeability but which is poorly quantitated, has been compared with fluorescein in parallel experiments.(4) In every instance in which an agent has produced a positive trypan blue response, the intensity of cutaneous fluorescein fluorescence was well above the control level. The same agents in dilutions not capable of producing a positive trypan blue response

did, however, show a relative increase in fluorescein intensity, implying that the fluorescein test is a sensitive indicator for intense as well as mild stimuli. It was noted frequently that agents which produced a positive trypan blue response, *viz.*: concentrated histamine, heat, topical bromobenzene in ether, etc., were unphysiological stimuli and may have injured the capillary endothelium, suggesting that such a test may be invalid as an indicator of permeability changes in the physiological range.

Accepting the presently held hypothesis that trypan blue is an indicator of increased capillary permeability, and that fluorescein behaves similarly in that both dyes appear extravascularly in greater concentrations under identical experimental conditions, it may be deduced that the intensity of cutaneous fluorescein fluorescence may be dependent to some extent upon permeability changes. It is evident that a rigorous experimental demonstration must be provided before a permeability factor can be read into the cutaneous fluorescein intensity measurements in its pres-



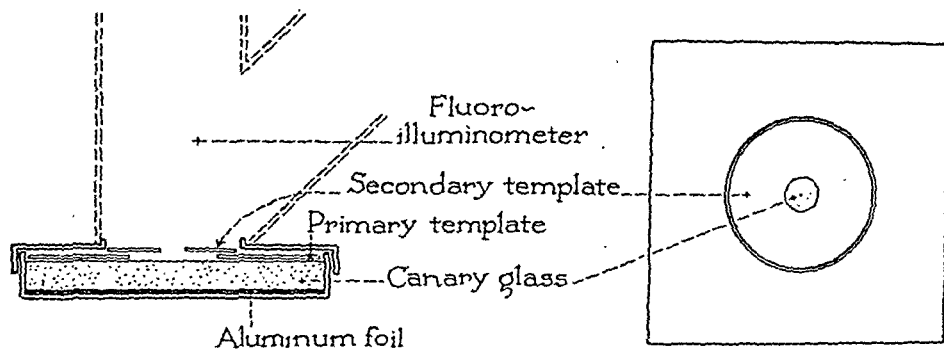


FIG. 2.  
Reference standard. (Not drawn to scale).

template of black heavy paper with a central aperture 18 mm in diameter covers the entire upper surface of the canary glass. A metal cover with a 4.6 cm diameter lipped aperture fits snugly over the reference holder and primary template to accommodate the base of the F-I in a light-tight gravity seal. A number of circular secondary templates having central apertures from 1-10 mm in diameter are cut from thin copper sheeting and coated with dull black lacquer. These discs have a slightly smaller diameter than the aperture in the metal reference standard lid so that they may be easily removed by simple inversion of the reference holder.

**Procedure.** The UV lamp in the fluoro-illuminometer is started approximately 10 minutes prior to the use of the instrument to ensure thermal stability and constancy of emission. The F-I is placed over the lipped aperture of the reference standard after insertion of a selected secondary template.<sup>††</sup> The phototube is zeroed for dark current and the sensitivity is adjusted to read 100% on the galvanometer scale. Prior to the administration of the fluorescein, the F-I is placed over the skin sites the fluorescence of which is to be measured and pre-dye readings are made which are later subtracted from each post-dye determination, thus compensating for inherent skin fluorescence. After each

measurement, or after a series of consecutive measurements, the F-I is again seated on the reference standard. A difference of more than 3% between the initial and final reference readings invalidates the measurement. If care is taken to ground and shield the equipment, and to select a UV lamp with stable characteristics, a series of 6 consecutive readings can be made in approximately one minute with reference variations of  $\pm 1\%$ . If the fluorescence from a small specific skin site, e.g. an intradermal wheal, is to be measured a blackened paper template about 2" x 2" with a central aperture corresponding to the diameter of the wheal is placed on the skin and the F-I centered on it. For more accurate centering on a discrete area, or when fluorescence is to be expressed as intensity per unit area, a prism finder head and iris diaphragm calibrated in terms of diameter can be fitted to the F-I. An accurate objective measurement of the circulation time of the dye is easily obtained by leaving the F-I on the skin while the dye is being injected. A sudden fluctuation of the galvanometer string indicates the exact moment of arrival of the dye at the skin site.

**Results and discussion.** The cutaneous vascular responses to a variety of chemical and physical agents employing the above procedure is under investigation. In most instances the vasoactive drug is prepared in serial dilutions and injected intradermally in the depilated abdomen of the rabbit, followed by the intravenous injection of sodium fluorescein (25 mg/kg in 5% aqueous solution). Fluorescent intensity over these sites

<sup>††</sup> The template selected is determined by trial and error. When the correct secondary template is chosen, it will usually be found that the entire range of fluorescence encountered can be read on the galvanometer scale without changing the shunt or phototube sensitivity.

TABLE I.  
 Effect, on Hatchability, of Vitamin B<sub>12</sub> Injections in Hatching Eggs.

Exp.	Class of embryo	Period of incub. at time of inj.	Level of B <sub>12</sub> inj. per egg,* $\mu$ g	Type of injections			Increase or decrease over H <sub>2</sub> O inj. eggs, %
				None Hatchability of %	H <sub>2</sub> O fertile eggs, %	B <sub>12</sub> %	
215	—	10 days	1.25	70.0	47.8	71.4	+23.6
220	—	10 "	1.25	75.0	82.8	80.0	— 2.8
228	—	Prior to incub.	0.50	—	74.4	83.3	+ 8.9
235	1	18 hr	—	83.5	—	—	—
	2	"	0.50	—	70.0	88.9	+18.9
	3	"	0.50	—	0.0	80.0	+80.0
240	1	"	—	86.6	—	—	—
	2	"	0.50	—	64.1	77.2	+13.1
	3	"	0.50	—	52.9	47.4	— 5.5
247	1	"	—	90.7	—	—	—
	2	"	0.50	83.3	68.9	76.9	+ 8.0
	3	"	—	45.9	—	—	—
252	1	"	—	85.5	—	—	—
	2	"	1.0	71.4	40.5	43.5	+ 3.0
	3	11 days	1.0	—	43.8	71.4	+27.6

\* Injected solution contained 25  $\mu$ g of vitamin B<sub>12</sub> per ml. An equivalent quantity of sterile distilled H<sub>2</sub>O was injected per egg in the H<sub>2</sub>O-treated groups.

of investigation of the role of vitamin B<sub>12</sub> in reproduction are presented herewith.

**Materials and Methods.** Because of the limited quantities of crystalline vitamin B<sub>12</sub> available, the effect of the vitamin was tested by injection into eggs rather than by administration to hens. All the hatching eggs used were laid by Rhode Island Red hens fed a diet deficient in vitamin B<sub>12</sub>.<sup>(4)</sup> The treated eggs were wiped clean at the large end (position of air cell) with cotton saturated with alcohol. A hole was drilled through the cleaned shell. Injections were made by means of a hypodermic needle into the albumen. Then the treated eggs were sealed with scotch tape and set in incubation trays. In all experiments there were control eggs injected with distilled water and in some experiments there were additional untreated controls. The number of eggs was limited by the supply of crystalline vitamin, and although the hens

were selected on the basis of a previous record of low hatchability, the sample of eggs used in the second experiment hatched too well to be suitable material.

Thereafter, eggs were selected for injection according to a method developed at this laboratory<sup>(5)</sup> for identifying eggs of low potential hatchability after 18 hours of incubation. By this method eggs are divided into 3 classes. Hatchability of fertile eggs in classes 2 and 3 is about 20% and 40% less, respectively, than in class 1. In most cases, eggs segregated in this manner were injected immediately after segregation, that is, after 18 hours incubation; but in one case the class 3 eggs were injected after 11 days incubation to permit removal of the considerable number of infertile eggs included in this class.

At hatching time all the chicks were wing-pedigreed and fed a 70% soybean meal basal diet<sup>(6)</sup> with and without a vitamin B<sub>12</sub> concentrate. The chicks were reared in

3. Lillie, R. J., Denton, C. A., and Bird, H. R., *J. Biol. Chem.*, 1948, v176, 1477.

4. Rubin, Max, and Bird, H. R., *Poultry Sci.*, 1947, v26, 309.

5. Olsen, M. W., *Poultry Sci.*, 1949, v28, 731.

6. Bird, H. R., Rubin, Max, and Groschlike, A. C., *J. Biol. Chem.*, 1948, v174, 611.

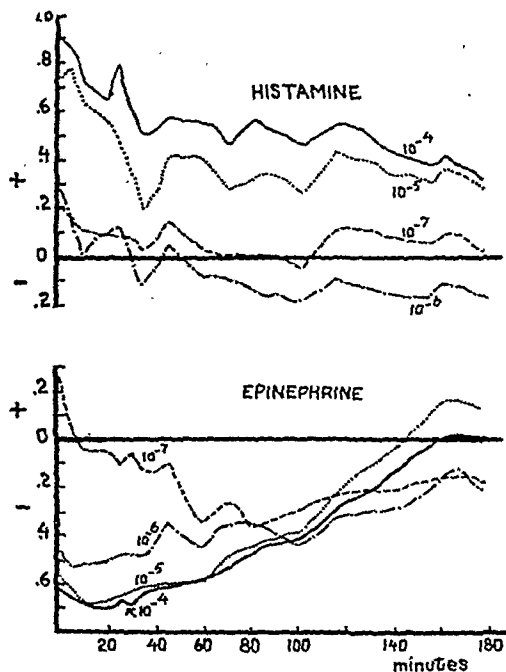


FIG. 4.

Fluorescein fluorescence time-intensity curves plotted as differences from the control saline wheal. fluorescence of drug wheal

Ordinate =  $\frac{\text{fluorescence of saline wheal}}{\text{fluorescence of drug wheal}}$  + = relative hyperfluorescence; - = relative hypo-fluorescence.

ent form. The rate of disappearance of fluorescein from the blood and the intact normal skin of the rabbit are closely parallel, (4,6) indicating that the altered time-intensity dye curves produced by the ad-

ministration of vasoactive drugs must be a property of altered capillary hemodynamics and/or a change in the permeability of the capillary wall. The exact factors responsible for the rate of dye exchange through the capillary wall can only be determined by the simultaneous measurement of these independent variables. The estimation of cutaneous fluorescein fluorescence as described above quantitatively indicates the gross rate of dye exchange across the capillary wall (neglecting lymphatic participation), but does not offer insight regarding the responsible specific factor(s).

*Summary.* 1. A quantitative method for measuring cutaneous fluorescein fluorescence is described.

2. The intensity of cutaneous fluorescein fluorescence is shown to parallel the expected capillary responses to various concentrations of vasoactive drugs.

3. The procedure described can be used to obtain in physiologically intact, unanesthetized experimental animals and probably human beings, a quantitative, sensitive and objective estimate of the cutaneous capillary circulation under a variety of experimental conditions.

The author acknowledges his indebtedness to Mr. Carl Griesmeyer for the skillful and intelligent construction of the apparatus described in this paper.

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### Role of Vitamin B<sub>12</sub> in Reproduction of Poultry.\* (17511)

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Research of the past few years showed that dried cow manure and certain animal protein supplements contained an unknown factor (or factors) required by chickens for growth and reproduction. (1,2) When crystalline B<sub>12</sub> be-

came available, it was found to possess activity for chick growth equivalent to that of the unknown growth factor occurring in cow manure and liver extract. (3) The results

\* Presented as part of symposium on vitamin B<sub>12</sub> and related factors, 116th meeting of American Chemical Society, September 22, 1949.

1. Rabin, M., and Bird, H. R., *J. Biol. Chem.*, 1946, v163, 357.

2. Whitson, D., Titus, H. W., and Bird, H. R., *Poultry Sci.*, 1946, v25, 143.

TABLE II.

Effect of Vitamin B<sub>12</sub> Injections in Hatching Eggs Upon Viability and Feathering of Chickens.

Exp.	Chick diet	Embryonic treatment	No. chicks		Mortality at 6 wk, %	Chicks with abnormal feathering, %	Feather score*
			At start	At end			
215	Basal	B <sub>12</sub>	30	22	26.6	36.3	—
		No B <sub>12</sub> †	34	13	61.7	84.6	—
220	Basal	B <sub>12</sub>	20	17	15.0	23.5	0.35
		No B <sub>12</sub> †	31	15	51.6	46.6	0.67
228	Basal	B <sub>12</sub>	18	11	39.9	27.3	0.55
		H <sub>2</sub> O	16	14	12.5	38.5	0.77
	+ B <sub>12</sub>	B <sub>12</sub>	17	11	35.3	9.1	0.09
		H <sub>2</sub> O	16	9	43.8	44.4	0.55
247	Basal	B <sub>12</sub>	10	7	30.0	0.0	0.00
		H <sub>2</sub> O	24	15	37.5	53.3	0.60
	+ B <sub>12</sub>	B <sub>12</sub>	10	7	30.0	0.0	0.00
		H <sub>2</sub> O	24	16	33.3	62.5	1.38
252	Basal	B <sub>12</sub>	17	14	17.6	21.4	0.21
		H <sub>2</sub> O	12	9	25.0	66.6	1.00
	+ B <sub>12</sub>	B <sub>12</sub>	15	11	26.6	27.2	0.45
		H <sub>2</sub> O	12	10	16.6	30.0	0.60

\* 0 = normal; 1 = slight abnormality; 2 = intermediate abnormality; 3 = extreme abnormality.

† Combined data from group receiving H<sub>2</sub>O injection and group receiving no injection.

known factor suffered a high mortality during the first week of life. This mortality occurred even when the chicks were fed a supposedly complete diet but was corrected by correcting the deficiency in the hens' diet. It has also been observed that the progeny of hens fed such a deficient diet show abnormal feathering which is not corrected by adding supplements to the chicks' diet.

The effects of B<sub>12</sub> injections in hatching eggs upon the viability and feathering of such chickens are summarized in Table II. With two exceptions, the mortality was greater for chicks hatched from H<sub>2</sub>O-injected and non-injected eggs than for those hatched from B<sub>12</sub> injected eggs, irrespective of chick diet. In experiments 215 and 220, in which 1.25 micrograms were injected on the tenth day of incubation, the B<sub>12</sub> injection reduced mortality very materially. Feathering of chicks hatched from B<sub>12</sub>-injected eggs was superior to feathering of those from the H<sub>2</sub>O-injected and non-injected eggs. As in earlier experiments addition of the vitamin to the diet of chicks hatched from deficient eggs

was not effective in improving feathering.

*Discussion.* The data summarized here indicate the importance of vitamin B<sub>12</sub> in reproduction of poultry, not only from the standpoint of hatchability but also from the standpoint of growth, viability and feathering of progeny. The effectiveness of a single dose of 0.5 to 1.25 µg of B<sub>12</sub> injected into hatching eggs in enabling the progeny to maintain an advantage in growth over control chicks for a period of 12 weeks is remarkable and indicates the high potency of the vitamin per unit of weight. The fact that injection of vitamin B<sub>12</sub> into deficient eggs reduced the mortality and improved the feathering of chicks and that these results were not achieved by supplementing the chicks' diet reemphasizes the importance of maternal diet and shows that deficient maternal diet may lead to irretrievable post-natal damage as well as pre-natal effects. Although vitamin B<sub>12</sub> reduced mortality and improved feathering, it was not completely effective with respect to either. This might well have been due to the very limited dosage, but of course there exists

electrically heated metal batteries with raised screen floors. Feed and water were supplied *ad libitum*. Body weights were recorded weekly, and feather observations at the termination of the experiments. All growth tests were of 6 to 12 weeks duration.

At the time of feathering observations, a feather scoring system was employed whereby those chicks with normal feathers were given the score "O." Those whose feathers were slightly frayed, thus having a rough appearance, were given a score of 1. Those whose feather tips were normal, but with an intermediate portion of the rachis free of barbs, were given a score of 2. Those with brittle, broken feathers were given a score of 3.

**Results. Hatchability:** The data on the effect of vitamin B<sub>12</sub> injections on hatchability are summarized in Table I. Since hatching eggs of class 1, representing embryos of most advanced development, were characterized by high hatchability, none of these eggs was used for injection work. Due to the fact that a very small number of eggs of class 3 was available, none was used as a non-injected control. As indicated in Table I, in the last column, 7 out of a total of 10 lots of injected eggs responded favorably to B<sub>12</sub>. Hence it is concluded that vitamin B<sub>12</sub> is required for hatchability of eggs laid by hens fed a diet deficient in the vitamin. Of the 3 lots that did not respond, one, composed of unsegregated eggs (Expt. 220), showed too high a hatchability without vitamin B<sub>12</sub> to permit any response, and another, the class 3 eggs in Experiment 240, constituted a small group in which the influence of unknown variables could easily have obscured the effect of the treatment.

**Growth.** The effects of B<sub>12</sub> injections in hatching eggs upon growth of chickens are summarized in Fig. 1. When the chickens were fed a basal diet deficient in the vitamin, all those that hatched from B<sub>12</sub>-injected eggs grew at a rate superior to that of the H<sub>2</sub>O-injected and non-injected groups for a period of 12 weeks. Since no differences were found between the H<sub>2</sub>O-injected and non-injected groups, the results from these two groups were combined. Fig. 2 shows the effect upon chick growth of different methods of ad-

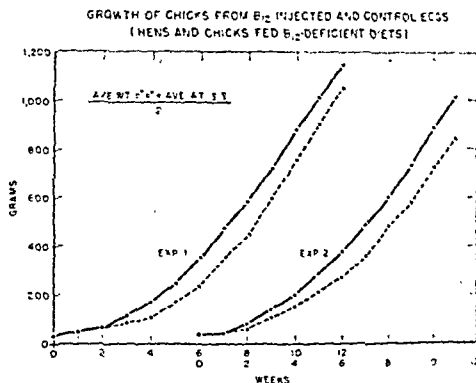


FIG. 1.

Growth of chicks from B<sub>12</sub>-injected and control eggs. Hens and chicks were fed B<sub>12</sub> deficient diets. Solid line, chicks from B<sub>12</sub> injected eggs. Broken line, chicks from eggs not injected with B<sub>12</sub>.

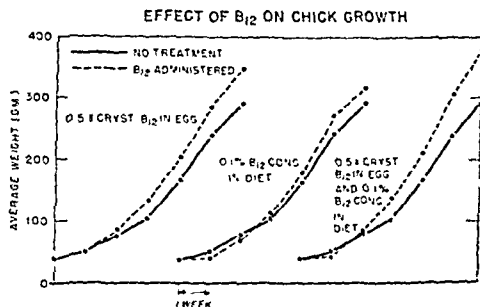


FIG. 2.

Effect on chick growth of vitamin B<sub>12</sub> injected into egg, added to diet, or administered by both routes.

ministration of vitamin B<sub>12</sub>. All the experiments in this case were of 6 weeks' duration. The first two curves are comparable to those shown in Fig. 1. The second two curves show that the growth response was greater for those chicks that received the supplemented diet than for those fed the basal diet alone when all chicks were hatched from eggs not injected with B<sub>12</sub>. As is shown in the remaining curves, the combination of dietary supplement with egg injection of the vitamin produced the greatest response.

**Viability and Feathering.** It was reported previously(7) that chicks hatched from the eggs of hens fed a diet deficient in the then un-

7. Bird, H. R., Rubin, Max, Whitson, Donald, and Haynes, S. K., *Poultry Sci.*, 1946, 25, 283.

TABLE I.

Recovery Values for Known Amounts of Chloramphenicol, a Hydrolysis Product, and a Glucuronide-derivative of Chloramphenicol Added to Human Blood.

Compound	Conc. in blood ( $\mu\text{g}/\text{ml}$ )	Whole blood		Red cells		Plasma	
		1:20 (%)	1:40 (%)	1:20 (%)	1:40 (%)	1:20 (%)	1:40 (%)
I. Chloramphenicol	80	81	92	75	87	90	94
II. Hydrolysis product	53	90	96	94	96	94	94
III. Glucuronide derivative	125	89	90	89	95	92	99

Dilutions of 1:20 and 1:40 were made in 3% trichloroacetic acid. The filtrates were analyzed for nitro compounds by the titanium reduction procedure. Values in the body of the table are given as per cent recovery, compared with those from aqueous solutions taken as 100%.

cal experiment a solution of purified I, II or III in physiological saline was added to heparinized blood and incubated at 38° with occasional shaking. Aliquots were withdrawn after 30 and 60 minutes, and a portion of each sample was centrifuged at 2100 r.p.m. for 15 minutes to separate the plasma and formed elements. The whole blood, plasma and red cells were then analyzed separately for aromatic nitro compounds, using the colorimetric procedure described elsewhere.(4) This method depends on reduction of the nitro group with titanous chloride, followed by diazotization and coupling with the Bratton-Marshall reagent.(5) The amount of color formed was found to be inversely proportional to the molecular weights of I, II and III. Samples were deproteinized with 3% trichloroacetic acid using a sample dilution of 1:40. Colorimetric standards were set up with the same solutions used for the experiments, and corrections were made for recovery from whole blood, plasma and red cells as described in the next section. Packed cell volumes were determined on the original blood specimens and on diluted blood taken at each sampling period. This was done by centrifuging in Wintrobe tubes at 2500 r.p.m. for 15 minutes, reading cell volumes, and then re-centrifuging to check the first reading. These figures were used for calculating the total content of nitro derivatives in the plasma and cells as a check on the analytical results.

**Results and discussion.** Data for the color-

metric recovery of known amounts of I, II and III added to whole blood, plasma and red cells are presented in Table I. The blood samples were diluted 1:20 and 1:40, using a final concentration of 3% trichloroacetic acid, and filtrates were analyzed for nitro compounds by the titanous reduction procedure.(4) From these results it is evident that dilutions of at least 1:40 must be used for satisfactory recovery of nitro compounds in the presence of red cells. These values were used to correct the analytical data obtained in the distribution experiments. The distribution of I between red cells and plasma was studied by the addition of 1925  $\mu\text{g}$  I in 6 ml of saline to 10 ml of heparinized blood. After standing at 38° with occasional shaking for 30 and 60 minutes, samples were removed for analysis as already described. In a similar manner, the amino diol compound (II) was studied by the addition of 640  $\mu\text{g}$  II in 3 ml saline to 10 ml of blood from a different individual. The glucuronide derivative was also handled similarly, with the addition of 2325  $\mu\text{g}$  III in 3 ml saline to 10 ml of heparinized blood. The results of these experiments are presented in Table II.

From these data it is evident that I and II are bound to a considerable extent by the formed elements of blood, while III is located almost entirely in the plasma with little or none present in the red cells. The binding is probably due to adsorption rather than slow diffusion through the cell membrane, since higher concentrations of nitro compounds are found in the red cells than would be expected on the basis of uniform distribution. This is also supported by evidence for the binding of

4. Glazko, A. J., Wolf, L. M., and Dill, W. D., *Arch. Biochem.*, 1949, v23, 411.

5. Bratton, A. C., and Marshall, E. K., *J. Biol. Chem.*, 1939, v128, 537.

the possibility that other dietary factors are involved.

**Summary.** When hatching eggs laid by Rhode Island Red hens fed a diet deficient in vitamin B<sub>12</sub> were injected with crystalline vitamin B<sub>12</sub>, the hatchability was improved.

Among the chicks hatched from vitamin B<sub>12</sub>-injected eggs, the growth rate was greater, the mortality lower and the feathering better than among chicks hatched from H<sub>2</sub>O-injected and non-injected eggs.

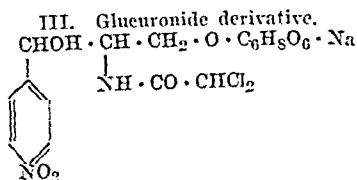
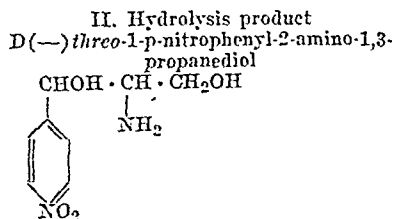
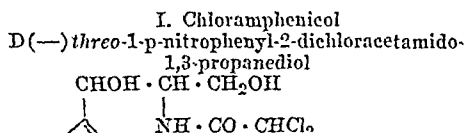
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## Distribution of Chloramphenicol (Chloromycetin\*) and its Metabolic Products Between Human Red Cells and Plasma. (17512)

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Following the administration of chloramphenicol to human subjects, 3 aromatic nitro compounds were identified as excretory products in the urine. (1) These were unchanged chloramphenicol (I), an amino diol produced from chloramphenicol by hydrolysis (II), and a conjugate of chloramphenicol with glucuronic acid (III):



Of these compounds only I showed a significant degree of antibiotic activity. The glucuronide derivative III constitutes the major excretory product in human urine, although small amounts of I and II are also present. (1,2) The reduction of the nitro group is negligible in man, although considerable quantities of aryl amines may appear in the urine and feces of lower animals. (2) The data presented here summarize our observations on the distribution of I, II and III between the red cells and plasma of heparinized human blood.

**Procedure.** The chloramphenicol used in these experiments was a crystalline product isolated from fermentation sources.† The amino diol was prepared by hydrolysis of chloramphenicol, (3) and the glucuronide derivative was isolated from human urine in this laboratory. (1) Blood drawn from the antecubital veins of normal human subjects was treated with a few milligrams of powdered heparin (110 units per mg) to inhibit coagulation, and was used immediately. In a typi-

2. Glazko, A. J., Wolf, L. M., Dill, W. D., and Bratton, A. C., *J. Pharm. Exp. Therap.*, 1949, v96, 445.

† The chloramphenicol used in these experiments was kindly supplied to us by Dr. John Ehrlich and Dr. Fred Stimpert. The amino diol (II) was prepared from chloramphenicol by Dr. Mildred Rebstock and Dr. Harry M. Crooks.

3. Rebstock, M. C., Crooks, H. M., Controulis, J., and Bartz, Q. R., *J. Am. Chem. Soc.*, 1949, v71, 2458.

\* Parke, Davis and Company trademark for chloramphenicol.

1. Glazko, A. J., Dill, W. D., and Rebstock, M. C., *J. Biol. Chem.*, 1950, in press.

force-feeding.(3) There is a simple method for the production of obesity in the rat, new in the laboratory, but representing a principle known throughout the history of medicine and animal husbandry. The development of obesity is related to the level of energy output as well as to the level of food intake. Restriction of the activity of the rat combined with the *ad libitum* eating of a diet which is appetizing to the animal, is a reliable means of promoting gains in weight in excess of the values regarded as standard for albino rats.

Male rats of the Sprague-Dawley strain were maintained on a stock diet of Archer Dog Pellets until they reached a weight of 325 to 375 g. They were then transferred to cages of one-half inch mesh screen 9 inches long, 4½ inches wide and 4½ inches deep. They were given a fluid diet to eat *ad libitum* (Table I). The room temperature was 74° to 78°F. Six rats were studied under these conditions. All of them gained in weight more rapidly than active stock animals or active animals on the same diet. We have never recorded a weight greater than 550 g

TABLE I.  
Medium Carbohydrate Diet.

Constituent	g
Cellu flour (Chicago Dietetic Supply)	120
Osborne and Mendel salt mixture	40
Diet yeast (Palbst)	100
Wheat germ oil	10
Cod liver oil	10
Vit. K (2-methyl-1,4-naphthoquinone)	100
Mazola oil	200
Casein (Labco)	160
Starch	200
Dextrin	190
Sucrose	200
	cc
Water to make total of	2000

3. Ingle, D. J., *Endocrinology*, 1946, v39, 43.

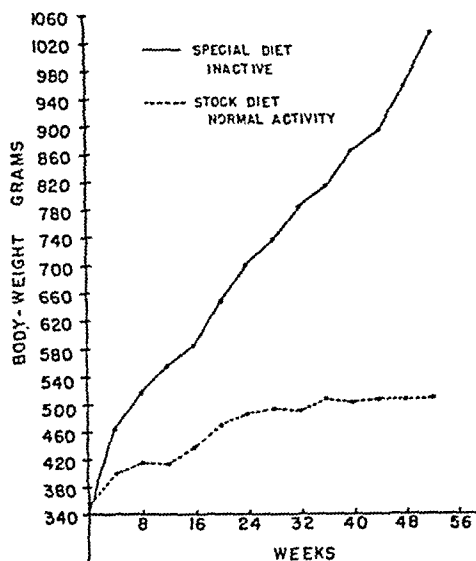


FIG. 1.  
Changes in weight of two normal rats.

among active adult male rats on the stock diet nor have we recorded a weight greater than 600 g for similar active animals fed the fluid diet in the large series of animals which we have observed during the past 10 years. After all of the 6 inactive rats were in excess of 700 g weight, 4 were killed for tissue studies, one reached a weight of 946 g and died and the 6th rat attained a maximum weight of 1090 g at which time it began to lose weight slowly and was killed for tissue studies. The weight chart of this animal is compared with the record of a typical active rat on the stock diet in Fig. 1. Insofar as we are aware, this is the greatest weight that has been reported for an albino rat.

**Summary.** Obesity can be produced in the albino rat by restriction of activity and by the *ad libitum* eating of a diet which is appetizing to the animal.

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TABLE II.  
Distribution of Nitro Compounds Between Plasma and Red Cells.

Compound	Theoretical conc. ( $\mu\text{g}/\text{ml}$ )	Incub. (min.)	Packed cell vol. (%)	Conc. of nitro compounds			Ratio: red cells/plasma
				Plasma ( $\mu\text{g}/\text{ml}$ )	Red cells ( $\mu\text{g}/\text{ml}$ )	Whole blood ( $\mu\text{g}/\text{ml}$ )	
I. Chloramphenicol	120	30	32.6	100	153	123	1.53
		60	32.6	98	157	118	1.60
II. Amino diol hydrolysis product	49	30	31.1	39	61	47	1.56
		60	33.7	33	68	46	2.06
III. Glucuronide derivative	179	30	39.3	274	55	168	0.20
		60	40.6	290	38	172	0.13

Plasma, red cells and whole blood were analyzed for nitro compounds following equilibration at 38° for 30 and 60 minutes, as described in the text. Values were corrected for recovery from each fraction as indicated in Table I. The ratios of concentrations found in red cells and plasma are given in the last column.

I to serum albumin.(6) In most cases, the amount of drug accounted for by the separate analysis of plasma and cells fell within 5 per cent of the actual amount of drug added. The observations presented here may assist in the interpretation of published data on the renal excretion of chloramphenicol and its inactive metabolic products.(2) The excretion rate for the glucuronide derivative III is no doubt greater than if it were bound to the cellular elements of blood, while the excretion of unchanged chloramphenicol is probably retarded by adsorption onto the erythrocytes, which would lower the effective concentration of freely diffusible drug in the plasma. The binding of chloramphenicol on the red cells, and pos-

sibly on the cells of other tissues, may constitute a reservoir of antibiotic which is in equilibrium with the body fluids. The existence of a different mechanism for producing active chloramphenicol by enzymatic hydrolysis of the inactive glucuronide has been described elsewhere.(2) These two factors may well be responsible for the maintenance of therapeutic levels of chloramphenicol in the blood stream over long periods of time.

*Summary.* Chloramphenicol and one of its hydrolysis products appear to be partly bound to the cellular elements of blood, while the glucuronide derivative of chloramphenicol remains almost entirely in the plasma. Observations are also presented on the recovery of these aromatic nitro compounds from trichloroacetic acid filtrates of plasma, cells and whole blood by colorimetric methods.

6. Vandenberg, J. M., cited by Smith, R. M., Joslyn, D. A., Grubitz, O. M., McLean, I. W., Penner, M. A., and Ehrlich, J., *J. Bact.*, 1948, v55, 425.

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## A Simple Means of Producing Obesity in the Rat. (17513)

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The effects of overfeeding and of obesity have been little studied in the laboratory. Voluntary hyperphagia in rats has been

stimulated by insulin(1) and by lesions in the hypothalamus.(2) In this laboratory, we have produced marked obesity in rats by

1. MacKay, E. M., and Cullaway, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1937, v36, 406.

2. Hetherington, A. W., and Ranson, S. W., *Anat. Rec.*, 1940, v78, 149.

TABLE I.  
Rate of Respiration of Chicken Erythrocytes in the Presence of Various Bacterial Exotoxins.

Cells exposed to equal volumes of:	$\mu$ L. O <sub>2</sub> /100 cells/hr												
	Ringer Loeke			Heated toxin (75-82°C, 75 min.)			Formalized toxin (0.02 M formalde- hyde 2 wk at 37°C + 2 mo. at 6°C)			Toxin or preservative			Remarks
	1st hr	2nd hr	3-5th hr	1st hr	2nd hr	3-5th hr	1st hr	2nd hr	3-5th hr	1st hr	2nd hr	3-5th hr	
1:20,000 phenyl mercuric acetate	9	10								9	11		No effect
1:10,000 merthiolate	9	10								9	10		" "
<i>Cl. perfringens</i> —strength unknown	9	9	8*	11	11		3	3	2*	14	7	0*	Initial acceleration, partial inhibition; complete inhibition†
<i>Cl. septicum</i> —160 MLD for mouse/cc	11	11		11	11		<1	<1		8	9		Little or no effect
<i>Cl. tetani</i> —300,000 MLD for guinea pig/cc	10	10	10†	13	14	13†	6	5		13	14	13†	Possibly slight acceleration
<i>B. cereus</i> —strength unknown	9	9		11	10		1			15	15		" "
<i>M. aureus</i> —15,000 dermal necrotic doses/cc	11	10	10	10	9	9	6		4	18	4	0	Initial acceleration, marked inhibition; complete inhibition†
<i>C. diphtheriae</i> —800 MLD for guinea pig/cc	12	12	12	10	8		5		2-3	20	7	4-3	Initial acceleration, partial inhibition, marked inhibition
<i>Str. pyogenes</i> —strength unknown	11	11		3	3		<1	2		3	<1		Marked inhibition, almost complete inhibition

\* 6-7 hr. † 3-4 hr. ‡ Possibly result of hemolysis.

# Action of Bacterial Toxins on Respiration of Chicken Erythrocytes.\* (17514)

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In the first paper of this series (Hunter and Larsh)(1) a brief statement of the general problem which is being investigated in this laboratory was presented. The present experiments were designed to demonstrate what effects, if any, a number of bacterial exotoxins have on the functioning of cells as indicated by changes in respiration.

The toxins were obtained from the Lilly and Lederle Laboratories<sup>§</sup> with the exception of *B. cereus*. This organism was grown in Bacto proteose peptone No. 3 broth and incubated at 37.5°C for 36-60 hours. The fluid suspensions were filtered through Seitz or Berkefeld filters and stored at 4°C with the relative humidity controlled at 95-100%.

A suspension of erythrocytes was obtained by centrifuging heparinized chicken blood. Equal volumes of cells and toxin, cells and heated toxin, cells and formalized toxin, and cells and preservatives (phenyl mercuric acetate or merthiolate) were mixed. Respiration measurements were made at 37°C, using a Warburg apparatus. One or 2 cc of the various cell mixtures were added to each Warburg vessel which contained 0.3 cc of 20% KOH in the center well. The time which elapsed from the mixing of the suspensions until the first reading was made varied from 15 minutes to an hour. Readings were started after a 10-minute period for tempera-

ture equilibration and were continued for varying periods of time up to 25½ hours. Sterile technics were used throughout and tests for contamination using agar slants and fluid thioglycollate were made at the end of each experiment.

The results are summarized in Table I, which also includes information concerning the relative strength of most of the toxins. It can readily be seen that formalized toxins are of no value as controls since formalin markedly inhibits the respiration of these cells. Except for the case of tetanal and streptococcal toxins, the heated toxins give essentially the same values as the Ringer Locke controls. Since the toxins were not highly purified, the conclusions which follow are tentative and may well have to be modified when experiments using purified toxins are available.

**Summary.** 1. Chicken erythrocytes were exposed to various bacterial toxins for several hours and the rate of oxygen consumption was measured at 37°C.

2. Toxins from *Clostridium perfringens* appeared to increase the rate of oxygen consumption initially even though they hemolyzed the cells. After several hours a decrease was observed.

3. Toxins from *Clostridium septicum* had little effect on the rate of oxygen consumption.

4. Toxins from *Clostridium tetani* apparently increased the rate of oxygen consumption for a period of more than 24 hours.

5. Toxins from *Bacillus cereus* had little effect, but slight acceleration of oxygen consumption was noted in most of the experiments.

6. Toxins from *Micrococcus pyogenes*, Var. *aureus* increased the rate of oxygen consumption during the first hour. This was followed by hemolysis of the cells and almost complete inhibition of respiration.

7. Toxins from *Corynebacterium diphtheriae*

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1. Hunter, F. R., and Larsh, Howard W., *Proc. Soc. Exp. Biol. and Med.*, 1947, v66, 281.

<sup>§</sup> The authors are indebted to the Lilly Laboratories and to the Lederle Laboratories for supplying these toxins.

stant weight at 95° C. Its globulin was determined by precipitating with equal parts of 4 M potassium acid phosphate solution (by the method of Butler), (7) centrifuging, and redissolving in physiological saline, then dialyzing against distilled water until chloride- and phosphate-free. At completion of the dialysis the globulin had reprecipitated. Albumin was determined by precipitating from the supernatant urine, after the globulin was precipitated, with an equal part of 20% trichloroacetic acid, centrifuging, resuspending the precipitate in water, and dialyzing until chloride- and phosphate-free.

B. *Evans blue (T-1824) determinations.* Evans blue forms a complex with plasma proteins. This property is made use of in the present experiments to label plasma proteins in their passage through the kidneys. Rawson (8) ascertained that, at pH 7.4, in solutions of human or canine plasma albumin, up to 14 molecules of Evans blue are linked to one molecule of plasma albumin. The greatest stability of the dye-protein complex is attained up to ratios of 8 molecules of Evans blue per molecule of plasma albumin. At such concentrations, the dye is wholly and preferentially bound by the albumin fraction, and in increasing proportions as the concentration of the dye increases. If the dye concentration is increased sufficiently, the dye may also be bound by the globulin fraction, but preferentially by the alpha-globulin. That, in the equilibrium system between the protein-dye complex and the free dye and plasma protein, the concentration of free dye is infinitesimal, is indicated by the observation of Rawson (8) that ultracentrifugation of a solution of dye-protein complex centrifuges down a blue-stained layer and leaves an unstained supernatant. Trypan blue is also bound by plasma protein; this complex is more unstable than the Evans blue-protein complex. When the trypan blue-protein complex is dialyzed in a cellophane bag the trypan blue is separated from the protein and stains the

cellophane. Evans blue, however, remains bound to protein under such circumstances, and leaves the dialyzing membrane colorless. The Evans blue molecule has 4 sulphonic-acid groups, and has a molecular weight of 960. The attachment of several such dye molecules to a molecule of plasma protein modifies the charge and the weight of the colloidal particle, and the addition of Evans blue to a solution of albumin has been found to lower appreciably the isoelectric point of the protein. (8)

A series of 8 male rats were given intracardiac injection of 0.5 ml of 5% Evans blue solution. The blood volume of the rat is reported as 4 ml per 100 g body weight, (9) of which approximately one half is plasma. Plasma protein concentrations are 5 to 7% (10) with about a 5 to 2 ratio of albumin to globulin. (10) It is readily calculated that, in the dose administered, all the dye combined with the plasma proteins. Starting 3 hours after administration of the Evans blue, urine was collected for 24 hours, observed qualitatively for color, and tested for protein with heat and acetic acid. The animals were then decapitated and autopsied. Kidneys were formalin-fixed in two series, unstained and stained with hematoxylin and eosin respectively, so as to facilitate study of both the course of the dye and of any possible renal damage that might have resulted from the injected Evans blue. In order to observe *in vitro* formation of the dye-protein complex in protein-containing urine, one drop of 5% Evans blue solution was added to the 24-hour urine sample of each of 4 untreated normal rats, and within two minutes this urine was tested for protein, as above.

Next, a second series of 13 male rats received intracardiac injections of 0.4 ml of 5% Evans blue. These rats were all decapitated, 7 rats at 10 minutes after injection of Evans blue; and 6 rats, 90 minutes after injection. Kidney slices from each of these rats were hardened in formalin solution for

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increased the rate of oxygen consumption slightly during the first hour. This was followed by a gradual decrease in rate until after 11 hours, then there was complete inhibition.

8. Toxins from *Streptococcus pyogenes* markedly inhibited the rate of oxygen consumption although there was no hemolysis.

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## Studies on Proteinuria in the Rat. (17515)

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Most investigations of protein excretion in the urine have assumed that protein in quantity is an abnormal constituent of the urine. It has furthermore been commonly assumed that mammalian kidneys extract from plasma a protein-free fluid in each species. The existence of a normal proteinuria in the Slonaker strain of white rats has been described qualitatively by Addis.(1,2) Protein excretion has been studied by Smetana(3) by injecting dye-labelled foreign protein into the blood of several mammals and observed deposition of dyed particles within the lining cells of the proximal convoluted tubules. Oliver(4,5) used similar technics both *in vivo* and in perfused isolated kidneys, with similar results. Dock(6) injected Evans blue intraperitoneally in rats and subsequently observed dye deposition in the renal tubules. The present studies again demonstrate a normal proteinuria, both albuminuria and globulinuria, under a variety of environmental conditions, in the albino rat. The passage of plasma protein through the rat kidney is studied by labelling the native plasma protein with Evans blue, and examining, in fixed tissues, the disposition of the Evans blue-

protein complex.

*Procedures.* A. *Protein determinations.* Urine was collected from rats, weighing from 180 to 250 g each, while in individual metabolism cages. During periods of urine collection, none of which was longer than 24 hours, no food was present in the cage. The animals studied included 14 male animals of the Wistar strain, on "Rockland Rat Diet", and located in a constant temperature room at 27° C; 12 animals, 8 males and 4 females, of the Sprague-Dawley-Holtzman strain, at ordinary room temperature, that had been on a diet of fortified powdered milk for a month; and 8 male rats of the Sprague-Dawley-Holtzman strain that had been subjected to a constant air temperature of 4° C for 3 months.

a) The urine was collected individually, centrifuged clear, and tested for protein content by heating and adding acetic acid. The precipitate so obtained was spun down washed and tested by the biuret test, xanthoproteic test, charring, and Millon test.

b) Qualitative protein fractionations of 72-hour urine specimens from 9 normal rats were performed. Urine was saturated with ammonium sulfate and a precipitate was interpreted as albumin. Globulin was precipitated by half saturation with ammonium sulfate. In both instances the precipitates were tested with protein reagents as above.

c) Quantitative protein measurements were made on 24-hour pooled urine from 5 normal male rats. Total protein was determined by precipitating with trichloroacetic acid, centrifuging, dialyzing against distilled water at 5° C till chloride-free, and drying to con-

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TABLE I.

Distribution of Evans Blue in Kidney Sections Taken 10 Min., 90 Min., and 24 Hr After Dye Injection.

Structures observed	10 min.	90 min.	24 hr
Blue-stained casts	+	+	+
Diffuse stain of superficial surface of tubular cells	+	—	—
Granular stain of tubular cells superficial	+	+	+
Luminal half of cells	—	+	+
Throughout cytoplasm	—	—	+
Stained granules in tubular cells showing various filamentous and rod-shaped forms	+	+	Indeterminate
Blue-stained arterial and arteriolar elastic membranes	+	+	+

stain, but were passed through paraffin to make permanent sections. These tissues showed the densest aggregations of intensely blue-stained particles, so numerous as to make any distinction between massed particles and single filaments very dubious. In these sections the dye was fairly evenly distributed throughout the cell, from luminal border to basement membrane (see Fig.). Again, in these sections, blue-stained casts were observed both in the proximal tubules and in the collecting tubules. Of much interest is the finding that all kidney sections contained areas of unstained proximal tubules which were estimated to be at a comparable level along the tubules as were intensely stained regions of other tubules in the same section. Hence it would seem probable that the dye-protein complex was filtered through the glomeruli in high dilution, and partly reabsorbed in the proximal tubules, possibly through the intermediation of mitochondria. Sections of the liver showed blue-dyed granules in the Kupfer cells and also showed blue-stained droplets of varying sizes in the cytoplasm of the liver parenchymal cells. Adrenal parenchymal cells and cardiac muscle fibers were unstained by Evans blue.

**Discussion.** Proteinuria occurs almost uniformly in normal laboratory rats. About half of this proteinuria is globulinuria. Proteinuria is a much less common finding in humans, but it does occasionally occur in the absence

of demonstrable renal lesions.(11,12) An interesting parallel between rat and human proteinuria is the prevalence of tubular casts in the normal rat kidneys and the prevalence of tubular casts in the kidneys of humans known to have had gross proteinuria, whether frankly pathological or not.

The source of the urinary proteins, and the method of their passage into the urine, are two points of interest.

a. Source of the protein. The renal glomerulus has long been considered by many renal physiologists to be impermeable to plasma protein molecules. Such an attribute would make the capillaries of the glomerular tuft quite unique, as complete impermeability to protein cannot be ascribed to any other capillary bed. Thus, the human cerebrospinal fluid has a modicum of protein (20 to 65 mg %) with up to 6 mg of this as globulin. Warren(13) found the extracellular fluid of the human leg to contain 0.9% of protein. It is possible, of course, that the renal tubules secrete the urinary protein; but there has been no evidence adduced to this effect. That the protein in rat's urine is plasma protein is indicated by the passage of Evans blue into the rat's urine, as Evans blue is firmly and

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the preparation of frozen sections.

**Results. A. Urinary proteins.** Two male rats maintained in the constant temperature room at 27°C gave negative tests for urinary protein. The other 28 rats, including animals of the 2 strains studied, and animals maintained at the several above-mentioned temperatures and diets, showed proteinuria. The amounts of protein, as observed grossly by the relative degrees of turbidity or flocculation on treatment of the urine with heat and acetic acid, varied in all groups, except in the group acclimatized to cold, from a moderate turbidity to a heavy flocculent precipitate. Urine samples collected from the animals kept in the cold room all showed heavy flocculent precipitates. All urine specimens fractionated by ammonium-sulfate precipitation contained both albumin and globulin. The urinary proteins were determined as 6.6 mg of protein in an average urine quantity of 8 ml per 210 g rat per 24 hour period. As separately determined, 3.0 mg of globulin, and 3.3 mg of albumin were excreted per rat in this period. No attempt was made to estimate the variation of proteinuria in an individual, but there was an appreciable variation in the bulk precipitate in the same rat in several 24-hour qualitative protein determinations.

**B. Evans blue studies.** Several rats, not included in the numbers reported above, died within a few minutes of injection with Evans blue, presumably from cardiac or pulmonary injury. All the 24-hour urine samples of the first group of 8 rats injected with Evans blue were blue-stained. The urine of all these animals was positive for protein as it had been in each animal when tested before the dye injection. The protein precipitate in all instances showed blue, and, when allowed to settle, was found to have withdrawn the dye from solution, the supernatant retaining no visible tinge. Normal rat urines to which a drop of Evans blue was added *in vitro* had their proteins precipitated with heat and acetic acid; precipitates so obtained were blue, and the supernatant showed no blue stain. All injected animals were decapitated. On macroscopic examination, in 21 animals injected with Evans blue, the tissues were, to a greater or lesser degree, blue stained. The liver con-

tained the most deeply dyed tissue. Kidney sections showed heavily stained cortical papillae which were sharply demarcated from the lightly tinted medullary tissue. Selective staining of the cortex was most intense in the animals killed after 24 hours, and least apparent in the specimens taken ten minutes after injection of the Evans blue. Microscopic studies of the kidney tissues of Evans blue-treated animals revealed several findings (see Table). All fixed hematoxylin and eosin stained sections taken from animals even 24 hours after Evans blue injection showed no morphological renal damage.

(a) In frozen kidney sections cut from animals killed 10 minutes after injection, blue-staining granules were observed, all superficially distributed in the cytoplasm (that is, toward the tubular lumen) of proximal tubular cells of many nephra. Several sections contained proximal tubular cells with a diffuse blue stain toward their luminal surface, as well as a similarly distributed, granularly disposed stain. The glomerular and capsular spaces were uniformly and distinctly clear of visible material, blue or otherwise. Blue-stained casts were found in the proximal tubules and in the collecting tubules of all specimens.

(b) In frozen sections of kidneys removed from animals killed 90 minutes after Evans blue injection, blue-staining granules were again observed in the cytoplasm of proximal tubular cells. Now, however, no diffuse distribution of the blue dye was apparent. Blue-staining granules in the cytoplasm were distributed superficially as well as more deeply throughout the cells, although the stained particles seldom approached the basement membrane, and were more concentrated toward the lumen. Cells in this group of sections contained appreciably more dye than in the ten-minute group. Also, the dyed particles were not merely in the shape of irregular granules of varying size as in the 10-minute sections, but frequently had a filamentous and often branching appearance, such as to suggest the possibility that the dye had become adherent to mitochondria.

(c) Kidney sections of animals killed 24 hours after injection received no post mortem

reabsorption of an Evans blue-plasma protein complex, and, by inference, of normal plasma protein. Such tubular reabsorption possibly involves the mitochondria of the tubular cells.

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## Nutritional Value of Plant Materials. II. Prevention of Acute Uremia of the Newborn Rat by Vitamin B<sub>12</sub>.<sup>\*</sup> (17516)

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A very high incidence of mortality during the first 24-72 hours of the life of young rats born to mothers maintained on rations containing a commercial soybean protein preparation and DL-methionine as the only source of amino acids has recently been reported from this laboratory.(1) In about 50% of the litters cast, from one to all of the young develop a characteristic syndrome terminating in early death. At birth and for the first 24-36 hours these young, on visual inspection, appear to be in every respect normal, indistinguishable from those which survive and develop normally. They are strong and nursed by their mothers until the onset of the crisis. (The support of excellent lactation has been an outstanding feature of these rations). A rapid onset of dyspnea accompanied by cyanosis, weakness and emaciation is typical of the syndrome observed. Milk is always present in the stomachs of the young and very frequently one or two loops of the small intestine contain dark material which is visible through the abdominal wall. At this stage the blood urea as determined by the method of Archibald(2) reaches values of about 150-250 mg per 100 ml as compared to about 35 mg at birth and about 50-70 mg in normal appearing rats of the same age.(3)

Death of these rats usually occurs within 4-6 hours after the first symptoms can be observed, although a few individuals have survived as long as 12 hours. Without inference concerning the nature of the biochemical or pathological lesion or the ultimate cause of death the syndrome is conveniently referred to as "acute uremia of the newborn."

Other investigators have reported early deaths of young rats(4-6) and mice(7) that had milk in their stomachs and they may have been dealing with the same syndrome. Zucker and Zucker(8) observed elevated concentrations of NPN and urea in the blood of rats of post weaning age which were fed rations devoid of animal protein. McGinnis *et al.*(9) refer to observation of an increased NPN concentration in the blood of chickens maintained on rations containing plant ingredients plus vitamin free casein. In this laboratory "acute uremia of the newborn" has never been observed on rations containing either alcohol extracted casein,(1) or a com-

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FIG. 1.

Kidney section of rat killed 24 hr after Evans blue injection ( $\times 400$ ). Glomerulus on right is unstained; some tubules are stained, others not.

preferentially bound to plasma protein. The data on Evans blue excretion must, however, be interpreted with reservations, as the protein-Evans blue complex represents an appreciable modification of the native plasma proteins, both as to molecular size and charge. Evans blue is a strongly acid substance and, as such, modifies the isoelectric point of the protein and might possibly modify the passage of the protein molecule across a membrane. There is also the possibility of a subtle effect of the Evans blue upon the kidney itself. However, no renal histopathology is noted after Evans blue injection.

b. Course of the protein from blood to urine. Evans blue appears earliest in the cytoplasm along the luminal borders of proximal tubule cells, and in intratubular casts, which are presumably protein in nature. Subsequently the dye pervades the cells and so approaches their basement membranes. This suggests that the tubules are absorbing the Evans blue, and, perhaps the protein with it, from the tubular lumen. If Evans blue did not pass into the tubular lumen by transfer across the tubular cells, the dye must have gained ingress by passing across the glomerular-capsular membrane, probably to be concentrated in the tubules into visible casts. Morphologically, the membrane appears to be non-secretory endothelium and would probably not disrupt the Evans blue-plasma protein complex. Also, the tubular

casts were stained blue, so that the Evans blue-plasma protein complex probably remains intact at least until it reaches the lumen of the tubule. Whether the tubular cells selectively absorb the Evans blue, leaving the protein free in the tubule, cannot be ascertained on the basis of the present experimental evidence. However, should unmodified plasma protein cross the glomerular-capsular membrane in the same manner as the Evans blue-protein complex, it would seem likely, by analogy with other blood-borne substances, as glucose and sodium, which are believed to cross the membrane freely, that the protein would be at least partially reabsorbed by the tubules. And similarly, it would then seem likely that the Evans blue is absorbed in the tubules in association with plasma protein.

The absence of Evans blue stain from the parenchyma of the adrenal and the muscle cells of the myocardium is in sharp contrast to the staining of kidney tubules and liver parenchyma. The latter two tissues are believed involved in plasma protein transfer, and it is therefore unlikely that the stain of these tissues is a non-specific or a general protoplasmic reaction. That the liver and kidney react diversely with the protein complex is indicated by the different dispositions of dye within the parenchymal cells of the two organs. The Evans blue-stained particulate material within the tubular cells could be interpreted as either an aggregation of the Evans blue-protein complex, or as a staining of existing cellular structures by the Evans blue. In this connection, the similarity in form and size of the intracellular aggregates of dye to that described for mitochondria is suggestive. The total absence of Evans blue from some proximal tubules, and the heavy staining of other proximal tubules, even 24 hours after injection of the Evans blue, indicates variation in activity among the nephra themselves.

*Summary.* Proteinuria was found to be a usual occurrence in albino rats, with an average excretion during fasting of 3.0 mg of globulin and 3.3 mg of albumin in 24 hours. Marked variation in the daily urinary protein excretion was observed. Evidence is obtained indicating glomerular filtration and tubular



FIG. 1.

Forty-four hour old rats; the 2 rats in the upper portion of the figure were injected 2 hours after birth with  $0.05 \mu\text{g}$  of vit.  $B_{12}$ ; the 2 rats in the lower portion are their uninjected littermates. The appearance of the latter is typical for "acute uremia of the newborn." Blood urea of these injected rats was 76 and 40 mg per 100 ml; that of the uremic control rats 287 and 240 mg per 100 ml.

syndrome together with their injected littermates is shown in Fig. 1.

The urea concentration in the blood of a few of the rats was determined 40-48 hours after birth. Others were kept to determine weaning weights. The results included in Table I show that rats with "acute uremia of the newborn" have extremely high blood urea values of about 200 mg per 100 ml, whereas normal appearing uninjected young or those injected with  $0.05 \mu\text{g}$  of vitamin  $B_{12}$  have a blood urea level of 50-60 mg per 100 ml. These levels fall into the range of values observed in a more extensive series studied in this laboratory(11) which revealed an early transient rise of urea in the blood of normal appearing young rats, even in those born to, and nursing, mothers maintained on the ration described here supplemented with 3% fish solubles or with 2% Wilson's liver extract 1:20. The present data suggest that either a single dose of  $0.05 \mu\text{g}$  of vitamin  $B_{12}$  is not sufficient to maintain the blood urea in the range of values found at birth or at weaning or that a transient rise may occur regardless of a vitamin  $B_{12}$  supply. The weaning

weights of the survivors were not significantly changed by the single injection of vitamin  $B_{12}$ .

The observations reported here do not explain the etiology of "acute uremia of the newborn" satisfactorily. Since the condition can be prevented by an early postnatal supply of vitamin  $B_{12}$  they suggest that the milk of the females maintained on the ration described here was low in vitamin  $B_{12}$  and that the fetal stores of this factor were likewise insufficient to overcome an early postnatal stress which may be associated with the adjustment of the newborn to the sudden supply of nutrients from the alimentary tract. The rapidity of its onset and its prevention by postnatal administration of vitamin  $B_{12}$  as well as the normal appearance of the young for the first 24 hours after birth further suggest that "acute uremia of the newborn" is of metabolic origin rather than a pathologic condition established *in utero*. Extensive data accumulated during experiments on reproduction of rats maintained on the ration described here show that often within a litter a few individuals show the acute syndrome while their littermates develop normally.(12) Furthermore, while a mother is maintained continuously on the same ration one litter may develop "acute uremia of the newborn" while the next litter does not, and vice versa. The incidence of the condition does not seem to increase greatly with successive generations. It appears therefore that under the conditions prevailing in these experiments circumstances of a marginal nature determine the incidence of the syndrome. Finally it has been observed(12) that a vitamin  $B_{12}$  deficient ration *per se* does not necessarily cause "acute uremia of the newborn." In view of these complex relationships clarification of the causes and of the nature of the syndrome must await further experimental evidence.

The acute uremia in newborn pigs reported by Madsen *et al.*(13) and the "toxic-uremic syndrome in baby pigs fed on dried skim milk" observed by Green *et al.*(14) may be related to the syndrome in rats described

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TABLE I.  
Effect of Vitamin B<sub>12</sub> on Acute Uremia of the Newborn.

	Inj. with 0.05 µg vit. B <sub>12</sub>		Uninjected controls	
	From 13 uremic litters	From 13 normal litters	From 13 uremic litters	From 13 normal litters
Total No. of young	46	54	52	63
Young dead in 48 hr from all causes	7*	4	35	5
Young with acute uremia	1	0	36	0
	Normal appearing young		Uremic young	Normal appearing young
Blood urea mg/100 ml	50.2 ± 11.8 (18)†		207.3 ± 58.2 (9)†	53.7 ± 19.8 (18)†
21 day weaning wt, g	Males 35.4 ± 3.1† (30)	Females 32.3 ± 3.7 (18)	Males 32.7 ± 5.4† (26)	Females 32.5 ± 5.2 (17)

Number of individuals given in parentheses.

\* Includes 3 young killed by female.

† Standard deviation. Uremic litters refers to those in which one or more rats were observed in acute uremic crisis. The others are referred to as normal litters.

mercial soybean protein supplemented with liver extract (Wilson's 1:20) or condensed fish solubles. Since the ration used in these experiments is presumably low in vitamin B<sub>12</sub> the experiments reported here were made. They appeared to be further justified by the preliminary observation that subcutaneous injection of an antipernicious anemia liver extract (Abbott) into the newborn rat prevented the development of "acute uremia of the newborn."

**Experimental.** The mothers of the young used for these experiments were fed the following ration: Commercial soybean protein (Archer Daniels Midland Co., Minneapolis) 274.4 g; DL-methionine, 5.6 g; salts 4(10) (Mn<sup>++</sup> concentration reduced to 1/10th) 40 g; vegetable fat (Crisco) 70 g; corn oil, 10 g; sucrose, 610 g. Each kg of ration contained in addition thiaminechloride HCl, 5 mg; riboflavin, 10 mg; pyridoxine hydrochloride, 5 mg; calcium pantothenate, 5 mg; cholinechloride, 1000 mg; i-inositol, 400 mg; para-aminobenzoic acid, 10 mg; nicotinic acid, 20 mg; 2-methyl-1,4-naphthoquinone, 5 mg; biotin, 0.2 mg; folic acid, 0.2 mg; d,l α-tocopherol, 100 mg; vitamin A (fish liver concentrate) 10,000 I.U.; vitamin D<sub>3</sub> (Delsterol)

1500 I.U. The mothers of the young used were F<sub>2</sub> or F<sub>3</sub> generation offspring from mothers on the same ration. They were kept on raised screens since weaning at 21 days of age except for a few days before parturition and during lactation. Within 2-3 hours after birth about half the number of live young in each litter were injected subcutaneously on the dorsal side with 0.1 ml of a solution containing 0.05 µg of crystalline vitamin B<sub>12</sub> (Cobione Merck). Before withdrawal of the needle the site of injection was covered with a small piece of adhesive tape to prevent leakage. The injected young were identified by snipping off the tip of the tail.

**Results and discussion.** A comparison of the incidence of "acute uremia of the newborn" in injected and uninjected littermates is given in Table I. Only those individuals were counted as having acute uremia which were actually observed in the acute crisis. Litters in which no acute uremia was observed are tabulated as "normal" litters. A few deaths in each group occurred from crushing by the mothers, from unknown causes or probably from unobserved acute uremia in the uninjected control group. The fact that one injected individual was observed in acute crisis suggests that the quantity of vitamin B<sub>12</sub> injected was not sufficient to prevent the syndrome in all cases. A typical example of the appearance of rats with the acute uremic

TABLE I.

Degrees of Equilibrium Attained with Various Durations of PAH Infusions without Priming Doses.

Patient PAH clearance PAH space	Time, min.	Plasma conc., mg %	Out/in ratio		
			Determined	Theoretical	Deter./theoret.
K.W.	83.2		.85	.86	.99
885 cc/min.	114	1.40	.90	.93	.97
37.1 L	143	1.45	.92	.97	.95
	175	1.47	.98	.98	1.00
J.D.	48.4		.82	.78	1.05
885 cc/min.	88.7	1.36	.87	.94	.93
28.0 L	112	1.44	.92	.97	.95
	141	1.46	.96	.99	.97
J.B.	37.1		.59	.63	.94
489 cc/min.	82.8	2.38	.89	.89	1.00
18.2 L	99.3	2.43	.88	.93	.95
	121	2.51	.95	.96	.99
	144	2.59	.95	.98	.97

As a criterion of equilibrium we have divided the rate of excretion in mg/min during a 15-20 minute period by the rate of injection in mg/min thus obtaining an "out/in ratio". As equilibrium is approached, this value approaches unity.

**Results.** Sixteen tests were done on 14 patients (all but 1 males, ages 11 to 50) and the volume distribution was calculated at 2 points in each test. The elapsed time at the first calculation point was 83 (68-121) minutes and at the second 130 (89-192) minutes. Most of the patients were excreting PAH in the urine at the same rate it was being injected (mean out/in ratio .96, S.D. .069, S.E. .017) by the end of the second period although not necessarily at the end of the first (mean .89, S.D. .094, S.E. .024). The mean apparent volume distribution at the end of the second period was 35.9% of body weight with a standard deviation of 7.3, a standard error of the mean of 1.8 and a range of 22.8 to 47.8. In terms of liters per square meter of body surface the mean was 13.4 with a standard deviation of 2.6, a standard error of the mean of 0.6, and a range of 9.1 to 17.7. All patients had priming doses except 3. Table I shows the time required in approaching equilibrium, in patients who received no primary dose. The percentage approach toward equilibrium is indicated by the out/in ratio (% of eventual plasma level is of

course an identical concept). In Table I the figures for time intervals and plasma levels correspond to the mid point of the 15 to 30 minute urine collection period used in calculating the observed out/in ratios, whereas in space calculations figures corresponding to the end of the urine collection periods were used.

**Discussion.** The figure 36% of body weight lies between those reported for extra cellular fluid—15% (7) to 25% (8) and total body water—53% (7) to 70% (9) indicating that PAH probably enters cells to some degree. It does not enter human red blood cells as several workers (10,11) have shown and we have confirmed. A recent statement (12) that human whole blood and plasma PAH concentrations are equal, was probably not intended and may have been an accident of wording. An alternative explanation to cell penetration might be that PAH reaches higher concentra-

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8. Kaltreider, N. L., Meneely, G. R., Allen, J. R., and Bale, W. F., *J. Exp. Med.*, 1941, v74, 569.

9. Moore, F. D., *Science*, 1946, v104, 157.

10. Smith, H. W., Finkelstein, N., Aliminos, L., Crawford, B., and Graber, M., *J. Clin. Invest.*, 1945, v24, 388.

11. Bradley, S. E., and Halperin, M. H., *J. Clin. Invest.*, 1948, v27, 635.

12. Cargill, W. H., *J. Clin. Invest.*, 1949, v28, 533.

in this paper. In view of the high early mortality observed in swine the prophylactic use of vitamin B<sub>12</sub> in these conditions should be investigated.

**Summary.** 1. Acute uremia of the newborn rat observed on rations in which a commercial soybean protein and DL-methionine furnish

14. Green, W. W., Kernkamp, H. C. H., Roepke, M. H., and Winters, L. M., *Am. J. Vet. Res.*, 1949, v10, 256.

the only source of amino acids can be prevented by subcutaneous administration of 0.05 µg of vitamin B<sub>12</sub> shortly after birth.

The assistance of Merck and Co., Abbott Laboratories, Wilson Laboratories, Lederle Laboratories, and DuPont and Co. with various supplies is gratefully acknowledged. Mr. Harlyn O. Halvorson kindly carried out the analyses for blood urea.

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### The Volume Distribution and Equilibrium Time of Para-Aminohippurate.\* (17517)

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Sodium para-aminohippurate (PAH) is widely used for measurement of effective renal plasma flow and maximal tubular excretory capacity (Tm). Its volume distribution is of importance in studies dealing with rate of change in PAH plasma levels and excretion following single injections, (1-3) or with constant infusions before equilibrium has been established. (4,5) Information concerning volume distribution is also useful in calculating optimum priming doses for rapid achievement of the desired plasma level. The literature pro-

vides very few data on the PAH space. It has been stated to be 28% of body weight in the dog (6) and estimates of 4-5 liters (4) and 20 liters (5) have been made for man. We are therefore reporting its determination in man.

**Methods.** All patients were free of renal, cardiac, or other diseases which might be expected to alter fluid compartments. An intravenous priming dose of PAH was given over a 7-9 minute period and this was followed by a constant intravenous infusion for 1½-3 hours. In three patients the priming dose was omitted. Infusions (both priming and sustaining) were given at an extremely constant and accurately known rate by means of a motor driven worm screw pushing on the barrel of a 50 cc syringe. Urine was collected by catheter with bladder washing. The anticoagulant was heparin powder. Total PAH was determined according to Newman. (4) This method in our hands gives complete hydrolysis of acetylated PAH. Its use, in the cases reported resulted in no destruction of free PAH, as indicated by identical values in infusion solutions analyzed as free and total. The total amount of PAH excreted was subtracted from the total amount injected and this difference divided by the plasma level to arrive at the apparent volume distribution.

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† Harrison Fellow in Surgical Research.

‡ National Institute of Health Post-Doctorate Fellow.

§ Woodward Fellow.

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2. Landowne, M., and Alving, A. S., *J. Lab. Clin. Med.*, 1947, v32, 931.

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4. Newman, E. V., Kattus, A., Genecin, A., Genest, J., Calkins, E., and Murphy, J., *Bull. Johns Hopkins Hosp.*, 1949, v84, 135.

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6. Houck, C. R., *Fed. Proc.*, 1949, v8, 78.

## A Rapid Bone Sectioning Technic.\* (17518)

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This paper describes a recently developed apparatus and technic for rapidly cutting undecalcified bone sections sufficiently thin for microscopic and autoradiographic study. This technic is particularly applicable to the sectioning of old, dried bone or bone from mature animals. Sections may be cut at the rate of 20 to 40 per hour and they may be cut serially with the loss of not more than 150 microns of material between sections.

**I. The Sectioning Apparatus.** A conventional brain microtome was adapted by substituting a rotary, motor driven saw blade for the stationary knife blade. The bone to be sectioned was clamped in the reciprocating carriage which fed the bone to the saw. The

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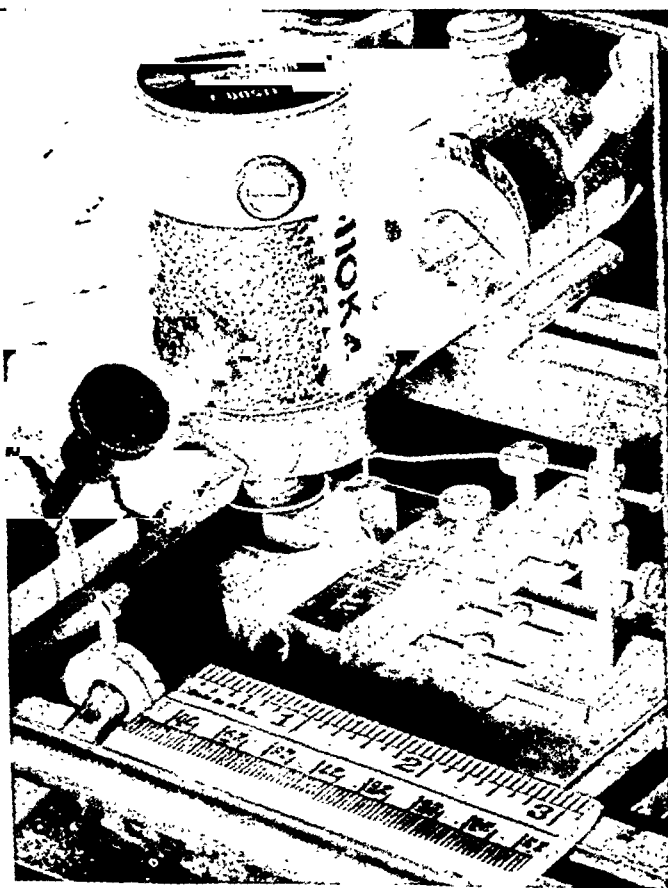


FIG. 1.

Bone-sectioning apparatus showing motor mounting and arrangement for clamping flexible plastic coverslip in contact with upper end of bone.

tions in some part of its volume distribution than in plasma. We have not considered in the present communication the question of expanding and shrinking apparent volume distributions. The mean space at the first calculation point was 34% and this rose to 36% by the time of the second calculation point (some 47 minutes later). This difference is statistically significant and could represent an expanding space. We are more inclined however to think that the finding arises from the fact that our priming dose in most cases was less than optimal and plasma equilibrium had not yet been achieved at the first calculation point. It is to be expected, of course, that any space calculation made before equilibrium has been achieved will be falsely low if the plasma level is rising and high if plasma level is falling. The magnitude of the volume distribution error is not known but it is probably small when the change in plasma level is occurring at a slow rate.

In practice of course, it is impossible to know what the eventual equilibrium plasma level will be and consequently one cannot detect the lack of equilibrium by comparing a specific plasma concentration with the eventual plasma level. It is known however what the eventual rate of excretion will be (*i.e.* equal to the rate of injection) and therefore examination of the out/in ratios is a far more useful index of equilibrium than judgments reached from comparison of several plasma determinations because, during a slow rise, the difference between successive samples may be no greater than the error of the method. For technical reasons equilibrium is desirable although not essential in all clearance determinations. If however, the rate of infusion is to be substituted for the rate of excretion,(5) then equilibrium is an absolute pre-requisite. Newman has suggested recently(4) that if the PAH prime be omitted entirely, equilibrium will be reached in 20-30 minutes. On the basis of out/in ratios however, our subjects did not seem to reach equi-

librium under 2 hours even with a priming dose. It can be shown by substitution of our determined PAH space and reasonable clearance values for the average person in Newman's formula<sup>1</sup> that, when the priming dose is omitted, it should require 77 minutes to reach 90% of the eventual plasma level, 100 minutes to reach 95% and 153 minutes for 99%. Table I shows experimental values in close agreement with these calculations. Newman's estimate of 20-30 minutes equilibrium time was based on an erroneously low value of 4-5 liters for volume distribution.

We have not attempted to determine experimentally the exact size of the optimal priming dose for rapid achievement of equilibrium. It would appear from comparison of our data with and without priming doses that use of doses of 0.5 to 0.7 g injected over a period of 7-9 minutes does not appreciably hasten equilibrium over complete omission of a priming dose. It may be that use of a considerably larger dose would be of value. Berger, *et al.*(5) seem to have had some success with priming doses of 0.8 g, frequently reaching satisfactory equilibrium in 60-90 minutes.

*Summary and conclusions.* 1. The apparent volume distribution of PAH is 35.9 (S.D.  $\pm$  7.3) % of body weight.

2. If the priming dose is omitted and the plasma level allowed to rise slowly during an intravenous infusion of PAH, the plasma concentration appears to reach about 90% of its eventual level in 75 minutes, 95% in 100 minutes, and 99% in 150 minutes in the average patient, so long as the plasma concentration remains below that of beginning saturation of the tubular excretory mechanism.

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$$P = \frac{IV}{C} (1 - e^{-Ct/Vc})$$

where P is the plasma concentration, IV is the infusion rate, C is the clearance, t is time and Vc is the apparent volume distribution.

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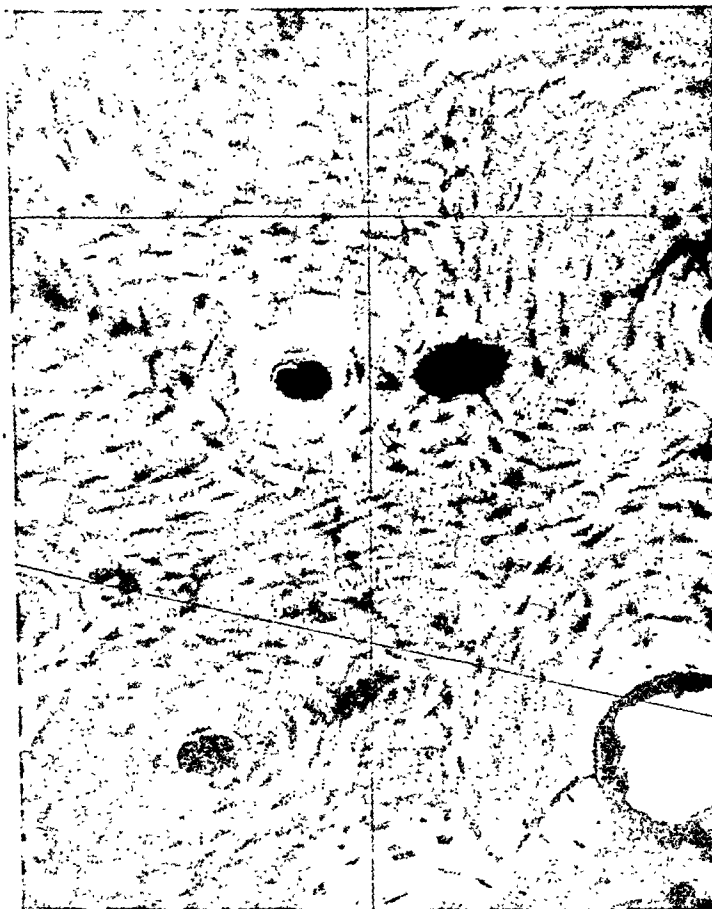


FIG. 3.

The cut section is approximately 30 microns thick and was cut from a piece of human bone from an individual 30 years of age at the time of death, which occurred 18 years ago.

of bioplastic which serves as a binder to prevent shattering of the section during cutting. The bone is prepared by embedding in Bioplastic<sup>§</sup> which, after hardening, is trimmed to leave only a thin shell surrounding the bone. This is best accomplished by using a router bit in a motor driven hand tool. A rectangular block of the plastic is left attached to the end of the bone by means of which it may be clamped in the microtome carriage.

**III. Sectioning Procedure.** Before cutting each section, a flexible, plastic coverslip is placed over the free end of the bone. The

coverslip is held in place by a holder mounted on a short metal rod, the base of which is attached to the carriage of the microtome.

A damper, consisting of a small piece of a microscope slide, is placed on top of that part of the coverslip immediately covering the free end of the bone. The damper, besides being attached to the coverslip holder by two strong threads, is held in place by a spring-wire keeper. The keeper is mounted in a position above the coverslip holder on the same rod. The end of the keeper is bent at right angles to the long axis and rests upon the damper. Fig. 2 is a schematic diagram of the arrangement of the various parts de-

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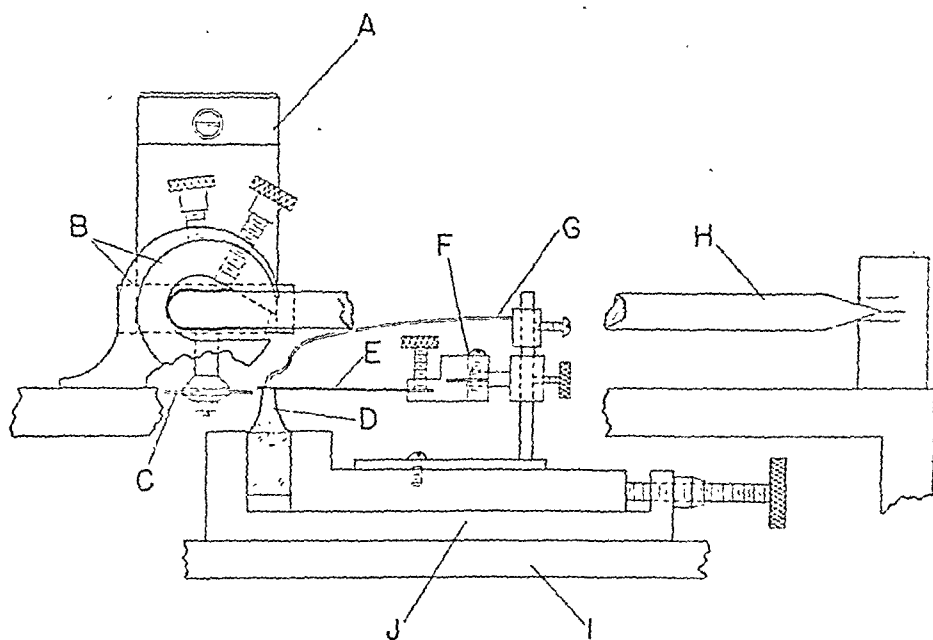


FIG. 2.

Details of bone-sectioning instrument. A. Motor. B. Motor mounting blocks which permit rotation of the motor about a horizontal axis perpendicular to the plane of the paper. C. Saw blade. D. Bone mounted in bioplastic. E. Flexible coverslip resting on upper surface of bone to retain cut section. F. Coverslip clamp. G. Wire for adjustment of pressure between coverslip and bone. H. Index arm for adjustment of attack angle of saw. I. Carriage way. J. Bone carriage.

motor used in this apparatus is a "war surplus" item<sup>†</sup> designed to drive a rotary switch in aircraft. Its small size ( $2\frac{1}{2}$  inches x  $1\frac{1}{2}$  inches diameter) and the rigid ball bearing mounting of the armature are highly desirable features. However, other designs of motors would no doubt be usable provided the armature end-play was eliminated. The motor is mounted on a solid steel bar machined to fit the knife blade clamps on the chassis of the microtome. These clamps are adjustable so that rotation of the motor about an horizontal axis perpendicular to the path of the bone is possible. This permits a fine adjustment of the angle of attack of the saw blade, preventing it from cutting out of or into the bone as the section is cut. Figure 1 is a close-up photograph of the essential features of the apparatus. The circular saw blades<sup>‡</sup> (commercially available for use in motor driven hand tools) are approximately  $\frac{3}{8}$  inch in

diameter, have 88 teeth without set and are 150 microns thick. The blades are attached to the armature shaft, the end of which is machined to the diameter of the hole in the blade, by clamping it between thick, cone-shaped washers which serve to hold the blade rigid. The speed of the motor is controlled by a variable transformer.

Since these blades are manufactured in quantity by stamping, it is frequently found that the mounting hole is not exactly centered. This results in excessive vibration which interferes with the cutting of the very thin sections. This difficulty may be overcome by placing the blade in a mandrel which holds it over an accurately centered hole slightly larger than the original hole. The mounting hole is then ground to the slightly larger size of the centered hole. This, of course, necessitates the use of a machined motor shaft the same size as the enlarged hole.

**II. Preparation of Bones.** The cutting of very thin sections (20-30 microns) necessitates that the bone be surrounded with a shell

<sup>†</sup> Bendix Radio, E. 11500-1, 28 Volts D.C.

<sup>‡</sup> Dremel Manufacturing Co., 2400 Eighteenth St., Racine, Wis.

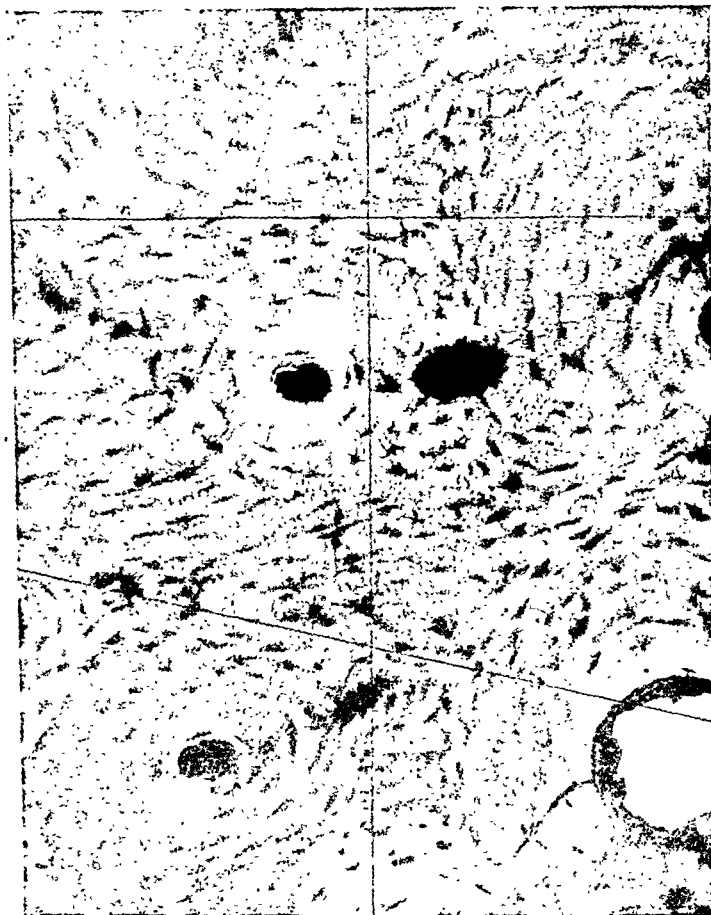


FIG. 3.

The cut section is approximately 30 microns thick and was cut from a piece of human bone from an individual 30 years of age at the time of death, which occurred 18 years ago.

of bioplastic which serves as a binder to prevent shattering of the section during cutting. The bone is prepared by embedding in Bioplastic<sup>§</sup> which, after hardening, is trimmed to leave only a thin shell surrounding the bone. This is best accomplished by using a router bit in a motor driven hand tool. A rectangular block of the plastic is left attached to the end of the bone by means of which it may be clamped in the microtome carriage.

**III. Sectioning Procedure.** Before cutting each section, a flexible, plastic coverslip is placed over the free end of the bone. The

coverslip is held in place by a holder mounted on a short metal rod, the base of which is attached to the carriage of the microtome.

A damper, consisting of a small piece of a microscope slide, is placed on top of that part of the coverslip immediately covering the free end of the bone. The damper, besides being attached to the coverslip holder by two strong threads, is held in place by a spring-wire keeper. The keeper is mounted in a position above the coverslip holder on the same rod. The end of the keeper is bent at right angles to the long axis and rests upon the damper. Fig. 2 is a schematic diagram of the arrangement of the various parts de-

<sup>§</sup> Ward's Natural Science Establishment, Rochester, N.Y.

scribed. The heat generated by the saw blade softens the plastic coverslip just enough to secure the section after the bone and surrounding plastic are completely cut through. The coverslip also prevents the section from warping. The damper serves to reduce a slight vertical flutter which appears in the saw blades when traveling at high speeds and also to minimize other extraneous vibrations. The keeper facilitates a fine adjustment of the pressure exerted upon the coverslip. The operating speed of the motor, and hence the saw blade speed, depends upon the type of bone being cut (degrees of hardness, inherent structure, etc.); the cross sectional area of the bone; the speed at which the bone is fed to the saw blade; and the thinness of the desired sections. Due to the extreme variability of these factors, it is difficult to set down definite rules for governing the saw blade speed; hence an insight into the problem must be acquired largely through experience.

The cut should be made in one firm smooth motion as rapidly as possible so that the section will be in contact with the saw blade a minimum period of time. It has been found that the bone should be moved against the

saw blade at a speed approximately 0.5 cm/sec. for best results. After cutting, the sections can be removed from the coverslip by means of a thin razor blade.

If sections are being cut from a bone having a relatively small cross sectional area, a section will sometimes be lost because not enough of the coverslip is softened to secure the section. This loss can be prevented by stopping the cut before the trailing edge of the plastic is completely severed. The cut can then be finished with a thin razor blade without loss of the section. Figure 3 is a photomicrograph of a section of human bone cut by this method. The cut section is approximately 30 microns thick and was cut from a piece of human bone from an individual 30 years of age at the time of death, which occurred eighteen years ago. The bones were kept in a sealed glass jar in the dry condition for the eighteen-year period. They were prepared for sectioning by embedding in Bioplastic as described above without any other treatment of any kind. Several thousand sections have been cut by this method.

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### Effect of Pilocarpine on Epinephrine Reversal Produced by 2-(N-p'toly-N-(m'-hydroxyphenyl)-aminomethyl)-Imidazoline. HCl (C-7337)\* (17519)

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The recent introduction of a number of new drugs, which antagonize the pressor action of epinephrine, has created renewed interest in the well known phenomenon of epinephrine reversal. This problem is of considerable practical importance since many of these newer agents are being used in the treatment of human disease as a possible substitute for surgical sympathectomy. There have been a number of hypotheses suggested which at-

tempt to explain the phenomenon of epinephrine reversal, none of which seems adequate. Substances which produce an epinephrine reversal are considered to block some part of the neuro-muscular apparatus which is concerned with the excitor actions of epinephrine, thus uncovering the inhibitory functions of epinephrine. The antagonism of epinephrine reversal by a wide variety of agents has been reported in recent months.(1-3)

\* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

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2. King, T. O., and Koppanyi, T., *J. Am. Pharm. Assn.*, 1949, v37, 346.

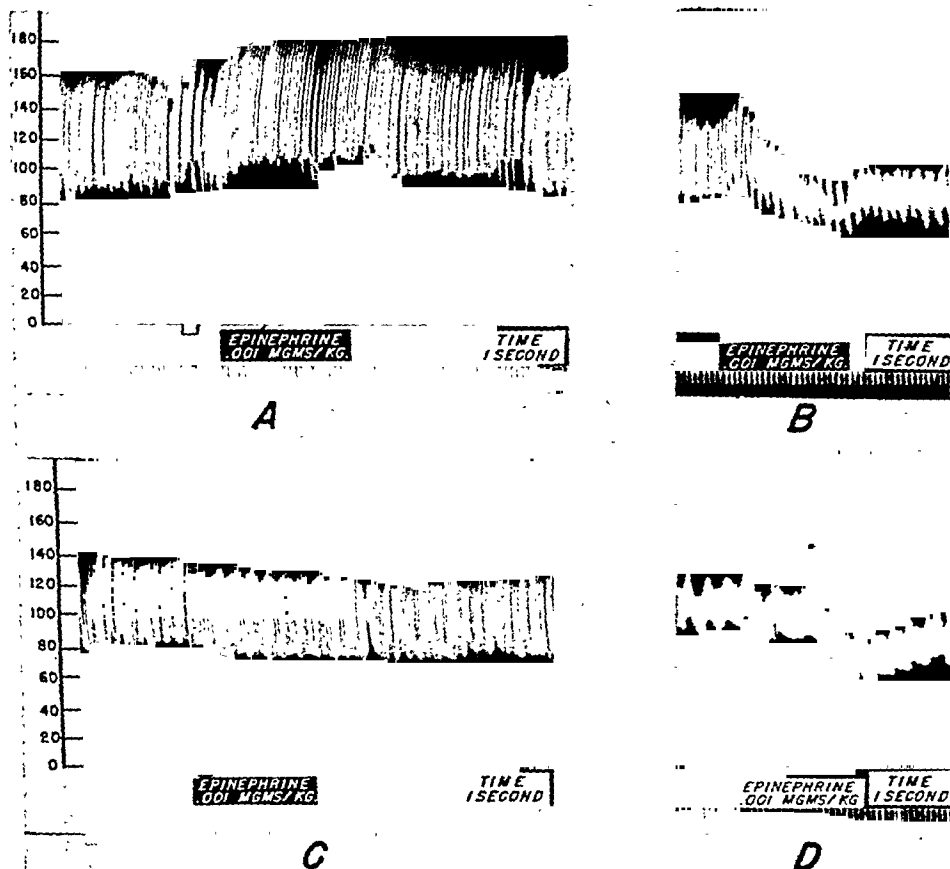


FIG. 1.

Unanesthetized dog. Events reading in sequence from left to right. Blood pressure recorded from femoral artery. A—Control injection of epinephrine. B—Epinephrine after 4.0 mg per kilo of C-7337. C—Epinephrine after C-7337 and 0.25 mg per kilo of pilocarpine. D—Epinephrine after C-7337, pilocarpine and 10 minutes after 1.0 mg per kilo of atropine.

Although there has been little similarity in the chemical or physiologic action of most of these agents, there has been some agreement that pilocarpine and other parasympathomimetic agents were most active. Since this phenomenon seems to us to have an important bearing on our concepts of autonomic physiology and pharmacology we have quantitated this effect using a new adrenolytic agent C-7337.(4)

*Methods.* Unanesthetized dogs were tied to

an operating table and under procaine local anesthesia, a femoral artery and vein were exposed and cannulated. The artery was connected to an Anderson glass capsule manometer(5) for recording of arterial pressure and pulse. The vein was connected to a system which permitted easy injection of drugs. All drugs were given intravenously and washed in with 10 ml of normal saline. Each animal was heparinized by the injection of 1 mg/kg of heparin and the manometer system was filled with heparinized saline. Recordings were made on smoked kymograph paper. Epi-

3. Konzett, H., *Experientia*, 1948, v4, 403.  
4. Meier, R., Yonkman, F., Craver, B. N., and Grass, F., *Proc. Soc. Exp. Biol. and Med.*, 1949, v71, 70.

5. Anderson, F., *J. Lab. Clin. Med.*, 1941, v26, 1520.

nephrine was injected in a dose which produced approximately 40 mm rise in pressure, following which 4 mg per kg of C-7337† was injected. This dose of adrenolytic agent is approximately 4 times that required to block the action of epinephrine(4,6) and produces effects which last for at least several hours. Following the injection of the adrenolytic agent, epinephrine was injected again in the same dosage as used in the control period. This invariably produced a fall rather than a rise in blood pressure. Gradually increasing doses of a variety of substances were then injected to test their effect on the epinephrine reversal. Dosages were continued until the epinephrine reversal was abolished or toxic symptoms were produced. Because of the fact that the injection of epinephrine after an adrenolytic agent produces an effect on the circulation comparable to that of isopropyl norepinephrine, it seemed of interest to determine the effect of pilocarpine on the depressor action of the latter compound.

**Results.** We have found that pilocarpine, physostigmine, and choline all possess the ability of abolishing epinephrine reversal. Kymograph records from a typical experiment are illustrated in Fig. 1. Since pilocarpine produced the most striking results, this agent was studied in sufficient detail to describe the

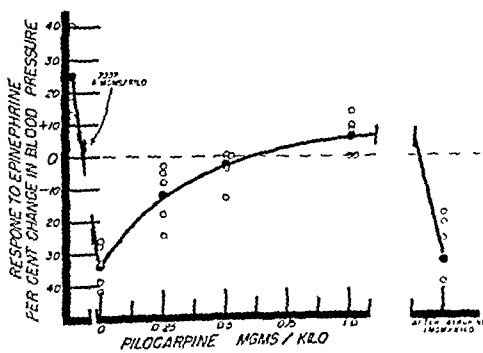


FIG. 2.

Summary of 5 experiments in unanesthetized dogs showing the relationship between dosage of pilocarpine and antagonism of epinephrine reversal by C-7337.

† Generously supplied by Ciba Pharmaceutical Products, Inc.,

6. Walker, H. A., Morrison, J. L., and Richardson, A. P., unpublished observations.

relationship between dosage and effect. A total of 5 dogs were used and the results of these studies are summarized in Fig. 2.

Following the abolition of epinephrine reversal, atropine in a dosage of 1 mg/kg was injected intravenously. Epinephrine injected within 5 minutes after atropine produced only slight effect upon blood pressure. If, however 10 minutes were allowed to elapse before epinephrine was reinjected, it invariably produced the same depressor effect which is observed immediately following administration of the adrenolytic agent.

In 6 experiments, using isopropyl norepinephrine, it was found that pilocarpine in a dosage of 0.5 mg/kg blocked the action of .001 to .004 mg/kg of isopropyl norepinephrine.‡ Fig. 3 illustrates one of these experiments. Of interest also was confirmation of the observations of Raymond-Hamet(7) that pilocarpine antagonizes the action of acetylcholine as shown in Fig. 4.

Further evidence of the similarity of action of isopropyl norepinephrine and the inhibitory effects of epinephrine following an adrenergic agent was shown by the fact that the pilocarpine blocking action could be reversed by atropine. Similar results have been reported by Fromherz.(8)

That antagonism of C-7337-epinephrine reversal by pilocarpine was not limited to this one adrenolytic agent was obtained from experiments showing that dibenamine-epinephrine reversal was also blocked by pilocarpine.

**Discussion.** The results presented above are difficult to explain in terms of present concepts of function of peripheral autonomic mechanisms. The similarity of action of isopropyl norepinephrine and the cardiovascular inhibitory actions of epinephrine after an adrenolytic agent suggests a common mode of action. The effect of parasympathetic stimulants and depressants on these phenomena suggest some relationship between the receptive mechanisms of the inhibitory sympathetic end organs and the receptive substance of the

‡ Generously supplied by Sterling-Winthrop Research Institute.

7. Raymond-Hamet, C. R., *C. R. soc. biol.*, 1934, v115, 1076.

8. Fromherz, K., *Experientia*, 1946, v2, 4.

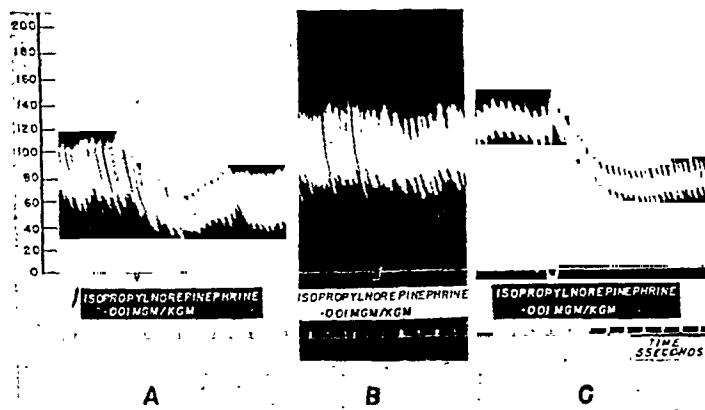


FIG. 2.

Unanesthetized dog. Events leading in sequence from left to right. Blood pressure recorded from femoral artery. A—Control injection of isopropyl norepinephrine. B—Isopropyl norepinephrine after 0.5 mg per kilo of pilocarpine. C—Isopropyl norepinephrine after pilocarpine and after 1.0 mg per kilo of atropine.

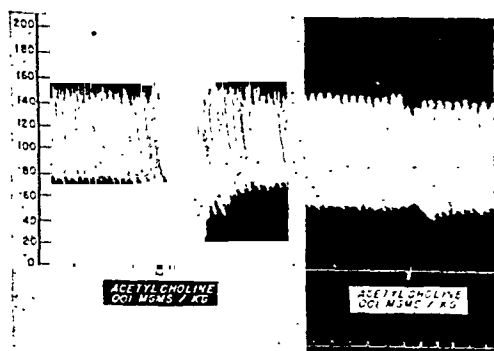


FIG. 4.

Unanesthetized dog. Blood pressure recorded from femoral artery. Left—Control injection of acetylcholine. Right—Same dose of acetylcholine 10 minutes after 1 mg per kilo pilocarpine.

parasympathetic system. This relationship, however, is not immediately clear, but points to the need for a more complete study of the interaction of autonomic drugs.

*Conclusion* 1) Epinephrine reversal produced by 2-(N-p'tolyl-N-(m'-hydroxy-phenyl)-aminoethyl)-Imidazoline HCl (C7337) may be abolished by the injection of pilocarpine and other parasympathomimetic agents.

2) The reversing action of such an adrenergic agent may be reinstated by injection of atropine.

3) Pilocarpine is also effective in decreasing the depressor action of isopropyl norepinephrine and of acetylcholine.

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# Chromatographic Demonstration of Histamine Release in Anaphylactic and Trypsin Shock. (17520)

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A large body of evidence has accumulated to indicate that histamine is released in anaphylactic shock and similar reactions in amounts adequate to account for many features of these phenomena.(1-4) The methods employed to ascertain the presence of histamine in such phenomena were based mostly on the biological activity of this substance. Convincing chemical proof has been lacking since adequately sensitive and specific chemical methods to detect small amounts of histamine in the presence of other substances have been developed only very recently.(5,6) A preliminary report has described the identification of histamine released from sensitized rabbit blood cells *in vitro* by paper chromatography(7) and further proof for the specificity of the method has been presented.(8) The purpose of this communication is to present more complete details as to the method and to report the results obtained by its use in anaphylactic and trypsin shock in dogs.

**Method.** Blood samples (40 ml) were collected in heparinized or oxalated tubes from dogs undergoing shock. The samples were centrifuged and the plasma (approx. 20 ml) separated. Each plasma sample was diluted with an equal volume of distilled water in a shell vial and 3 g of a salt mixture (consisting

of 62.5 g anhydrous  $\text{Na}_2\text{SO}_4$  and 10 g  $\text{Na}_3\text{PO}_4 \cdot \text{H}_2\text{O}$ ) was added for each 10 ml of the diluted plasma, according to the procedure of McIntire.(9) To each 20 ml of diluted plasma 10 ml of butanol were added to extract the histamine and the samples were shaken in a mechanical shaker for 30 minutes. The aqueous, protein and butanol layers were separated by centrifugation. The butanol layer was drawn off and placed into a clean shell vial. The butanol extraction was repeated and all butanol extracts from each plasma sample were combined. (An increase in the amount of butanol was not found to improve the histamine extraction significantly, with amounts of plasma up to 20 ml. Increasing the number of butanol extractions, similarly, had no appreciable effect on the recovery of histamine when added to control plasma.)

To the combined butanol extracts were added 5 ml of 0.5 N  $\text{H}_2\text{SO}_4$ . The tube was shaken for 10 minutes, centrifuged briefly and the aqueous bottom layer transferred to a small glass-stoppered vial by means of a suction-bulb pipette.(9) The butanol solution was again extracted with 2 ml of  $\text{H}_2\text{O}$  in a similar manner and this second aqueous layer combined with the acid.

The combined  $\text{H}_2\text{SO}_4$  and  $\text{H}_2\text{O}$  extracts were neutralized with 10% NaOH in a small glass-stoppered vial and 1.5 g of the previously mentioned salt mixture and 2.5 ml of butanol were added. After shaking the vial for 20 minutes, the layers were separated by brief centrifugation and the butanol layer transferred with the suction-bulb pipette to an applicator pipette in contact with the filter paper strip\* which was stretched horizontally

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\* The filter paper used in this and previous work was supplied by the Eaton-Dikeman Co., Mount Holly Springs, Pa., in convenient rolls,  $\frac{5}{8}$ " wide.

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TABLE I.  
Histamine Content ( $\mu\text{g}$  Base per 20 ml) of Dog Plasma Before and During Anaphylactic Shock.

Dog. No.	Degree of shock	By paper chromatography		By bioassay	
		Before	During	Before	During
1	Fatal	0	>10	<1	50
2	"	0	>10	<1	48
3	"	0	>10	<1	16
4	Mild	0	0	<1	<1
5	Fatal	0	>10	<1	28

TABLE II.  
Histamine Content ( $\mu\text{g}$  Base per ml) of Dog Plasma Before and During Trypsin Shock.

Dog No.	Dose trypsin, mg/kg	Degree of shock	By paper chromatography		By bioassay	
			Before	During	Before	During
1	50	Mild	0	>3<5		Not done
2	100	Fatal	0	>10	<1	38
3	100	"	0	0	<1	<1
4	100	"	0	>10		Not done
5	100	"	0	>10	"	"
6	100	"	0	>10	"	"

over a hot plate (temperature at level of strips 70-70°) as previously described.(10) The extraction of the aqueous layer was repeated with 1 ml of butanol which was added to the first butanol extract in the pipette.

After application of the final butanol extract to the paper strip, the strips from both control and shock samples of blood were stapled to narrow cardboard at intervals of  $\frac{1}{2}$  cm. Chromatography with butanol saturated with 10%  $\text{NH}_4\text{OH}$ , and color development with diazotized p-bromoaniline was carried out as described in a previous communication.(8) Histamine, when present in amounts exceeding 2-3  $\mu\text{g}$ , made its appearance on the strips as a red band at an  $R_f$  value of approximately 0.56. Control strips to which known amounts of histamine had been applied were run simultaneously. This permitted a rough approximation of the amount of histamine in the 20 cc plasma samples.

*Anaphylactic shock.* Blood samples were collected from 5 dogs sensitized to horse serum both before and 1-3 minutes after i.v. injection of the antigen. The release of histamine as shown by chromatography and bioassay† is summarized in Table I. One of the experiments is illustrated in Fig. 1.

*Trypsin shock.* Six dogs were injected i.v. with crystalline trypsin (Armour). Blood samples were taken before and during shock. The results are summarized in Table II. One of the experiments is illustrated in Fig. 2.

Release of histamine in dogs shocked with peptone could not be demonstrated by chromatography since the amounts of peptone required to produce severe or fatal shock (1 cc of 20% Bacto-Protone (Difco) kg) interfered markedly with the chromatographic procedures. This was in part due to the interference of peptone with proper separations on the chromatograms, as well as to the presence of substances in the peptone solutions which give a strong color reaction with the diazotized p-bromoaniline used as the color reagent on the chromatograms. Attempts to separate these interfering substances by either electrodialysis or repeated butanol extractions of peptone solutions resulted in fractions which did not produce shock in the dogs. Addition of small amounts of peptone solutions to normal rabbit blood, however, caused release of histamine into the plasma which could be demonstrated by the chromatographic procedure.

† All bioassays were kindly performed by Dr. Lorenzo Giscarfé and Dr. Wilson T. Beraldo.



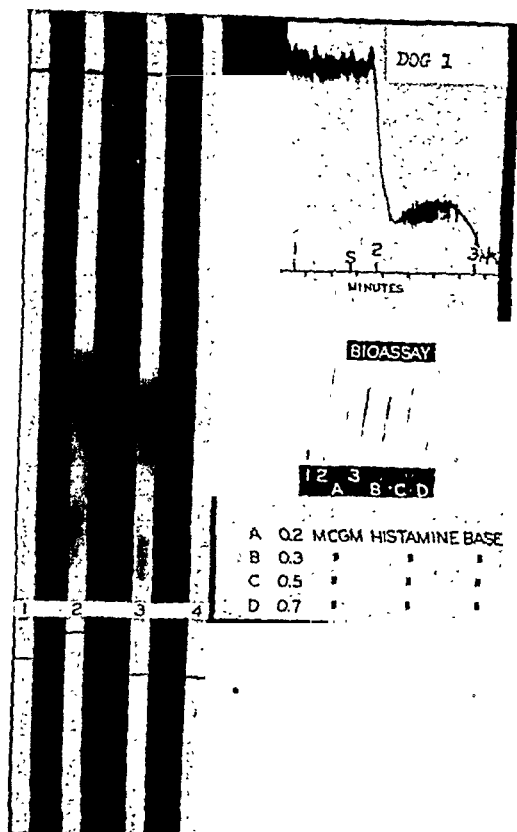


Fig. 1.

Paper chromatograms, blood pressure record and bioassay from a dog during anaphylactic shock.

1, 2 and 3 on B.P. record signify blood samples drawn S—antigen injection.

1, 2 and 3 on chromatograms and bioassay record correspond to the respective blood samples.

4 on chromatograms represents 10  $\mu$ g pure histamine base chromatographed as control. Note the much heavier bands of histamine on strips 2 and 3 at the same location and absence on strip 1. The substance(s) on strip 2 and 3 responsible for the dark bands below the histamine bands have not been identified.

A, B, C, D on bioassay record represent standard amounts of histamine added to the muscle bath for calculation of the amounts of histamine in the plasma samples.

**Discussion.** The experiments described identify histamine liberated during anaphylactic and trypsin shock in dogs. While the chromatographic method as employed routinely in this study does not permit exact quantitation, a better estimation of the amount of histamine can be achieved by

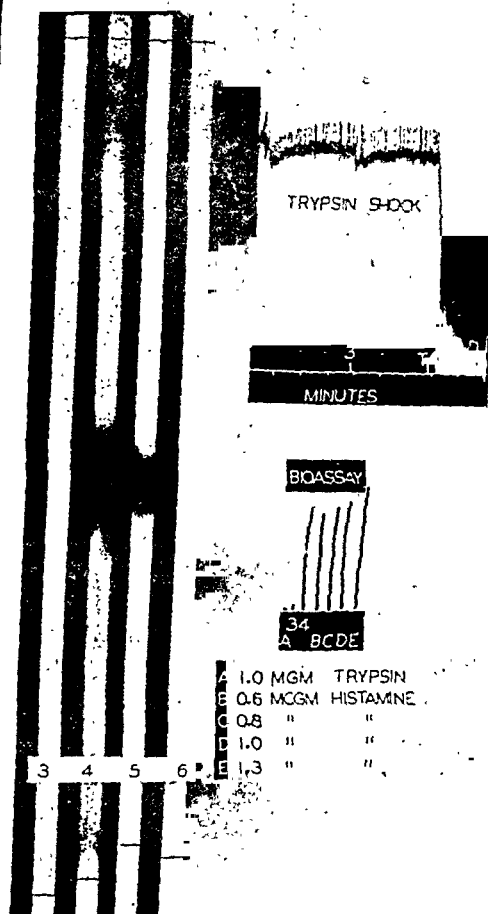


Fig. 2.

Paper chromatograms, blood pressure record and bioassay from a dog during trypsin shock.

3 and 4 on B.P. record signify blood samples drawn T—trypsin (100 mg/kg) injection.

3 and 4 on chromatograms and bioassay record correspond to the respective blood samples.

5 on chromatograms represents 10  $\mu$ g pure histamine chromatographed as control.

6 on chromatograms represents 40 mg trypsin chromatographed as control.

A on bioassay record represents a trypsin control.

B, C, D, E on bioassay record represent standard amounts of histamine added to the muscle bath for calculation of the amount of histamine in the plasma samples.

chromatographing a series of control strips with graded amounts of histamine along with aliquots of the sample. For example—ali-

quots of the first butanol extract of the shock plasma obtained from one of the dogs were chromatographed with a standard series of histamine samples. A butanol sample containing 7.8  $\mu$ g histamine (calculated on the basis of bioassay of the plasma) showed a chromatogram falling between standard chromatograms of 5 and 10  $\mu$ g histamine and corresponding most closely to the 7  $\mu$ g standard as judged by an unbiased observer. The chromatographic method with a limit of sensitivity of 2-3  $\mu$ g per sample is obviously inferior to bioassay in this respect but is superior so far as specificity is concerned. The demonstration of histamine release in amounts roughly equivalent to those observed in the anaphylactic experiments in a high percentage of the trypsin shock experiments confirms an earlier report from this laboratory(3) but is in contrast to a more recent report, also from this laboratory.(11) We are not at present

prepared to explain the discrepancy.

**Summary.** A method for the chemical identification of histamine in blood by paper chromatography is described in detail and experiments are presented to show the release of relatively large amounts of histamine in dogs undergoing anaphylactic and trypsin shock. Peptone, when injected into dogs in amounts sufficient to produce severe shock, interfered with the identification of histamine which could, however, be found on the chromatograms after addition of small amounts of peptone to rabbit blood (*in vitro*).

The author wishes to express his appreciation to Dr. Carl A. Dragstedt for many helpful suggestions.

11. Wells, J. A., Morris, H. C., and Dragstedt, C. A., *Proc. Soc. Exp. Biol. and Med.*, 1946, v62, 209.

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## Inhibition of Hyaluronidase by Aromatic Compounds. (17521)

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The aim of our experiments was to find substances inhibiting the action of hyaluronidase. Repeating Guerra's work,(1) we(2) confirmed his observations that this enzyme was inhibited by sodium salicylate. Prior to this, hyaluronidase was found to be inhibited by heparin, gastric mucin, pseudo-globulin,(3) morphine,(4) hyaluronic acid,(5) estrogens(6) and, of course, enzyme inactivators. These experiments were carried out *in vivo*. By studying the effect on the area

of spread in the skin, it was found that ascorbic acid,(3) glycerin,(7) triacetin,(7) arsenious oxide,(7) lecithin,(8) peptones,(8) urethane, sodium diazobenzene sulfonate,(9) hirudin,(7) human urine(10) and diazotized proteins(11) enhance this enzymatic action. Obviously, these lists fail to reveal chemical structures which are essential for anti-hyaluronidase activity. We, therefore, undertook to study a series of simple chemically related compounds and used the turbidimetric method described by Kass and Seastone.(12)

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2. Calesnick, B., and Beutner, R., *Fed. Proc.*, 1947, v6, 1.

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5. Meyer, K., Dubos, R., and Smyth, E. M., *J. Biol. Chem.*, 1937, v118, 71.

6. Sprunt, D. H., McDearman, S., and Raper, J., *J. Exp. Med.*, 1938, v67, 159.

7. Hobby, G. L., Dawson, M. H., and Meyer, K., *J. Exp. Med.*, 1941, v73, 109.

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9. Evans, D. G., *Nature*, 1940, v145, 866.

10. Christensen, J. F., *Hospitalstid.*, 1948, v81, 572.

11. Madinaveitia, J., *Biochem. J.*, 1941, v35, 453.

12. Kass, E. H., and Seastone, C. V., *J. Exp. Med.*, 1944, v79, 319.

It is based on the observation that pure hyaluronate in a buffered solution produces a fairly stable colloidal suspension when mixed with diluted acidified horse serum. There is a reduction in turbidity when hyaluronate is depolymerized by hyaluronidase. The efficiency of various inhibitors can be studied by measuring the turbidity of the various mixtures when compared to controls. This turbidity is a function of the undecomposed hyaluronic acid present. When the enzyme is introduced into the system, the turbidity produced holds an inverse relationship to the hyaluronidase content.

To determine the effect of a particular chemical, a series of freshly prepared dilutions of the enzyme were prepared. Five tubes were used with serial dilutions running in the order of 10, 20, 30, 40 and 50  $\gamma$  of enzyme per cc. Since the concentration of the original solution was 100  $\gamma$  per cc, 0.1, 0.2, etc. of this solution was placed in the tube and diluted to 0.5 cc with buffer pH 6.0 to maintain constant volumes. To each of these dilutions, 0.5 cc of the hyaluronate solution (0.04 mg/cc) was then added. Immediately after this, 0.1 cc of the compound to be tested was added to another tube which contained 50  $\gamma$  of hyaluronidase and the substrate. All tubes were then incubated for 30 minutes in a constant temperature water bath at 37.5°C. The remainder of the procedure was performed as outlined by Kass and Seastone. Whenever possible, several determinations were made the same day. Each day a curve was plotted anew. This was desirable because of the variability and colloidal instability of proteins in the diluted horse serum which served as an indicator for undecomposed hyaluronic acid present. All the curves were S shaped in form and were prepared by using the dilution as abscissa and the turbidity, in terms of the reading on the Klett-Summerson photo-electric colorimeter, as ordinate. Red filter No. 66 was used.

As an example, the curve obtained using sodium salicylate is illustrated in Fig. 1. Here the reading on the colorimeter corresponding to 50  $\gamma$  of hyaluronidase is 35. When 10 mg of sodium salicylate was added, the reading was 47, which corresponds to a

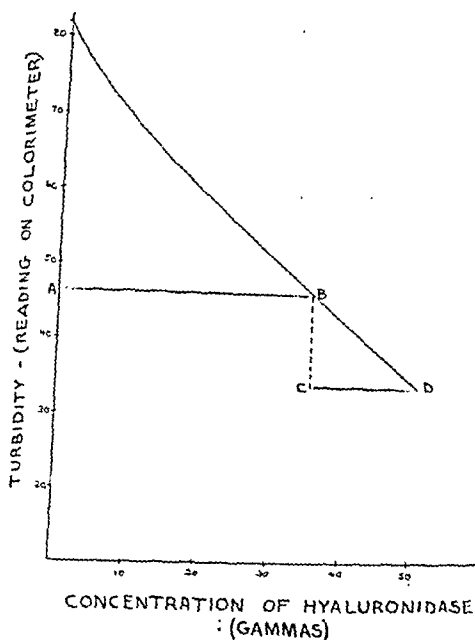


FIG. 1.

Method used to calculate activity of inhibitors.  
D—Turbidity equivalent to 50  $\gamma$  of hyaluronidase.

B—Turbidity obtained by the addition of sodium salicylate.

AB—Quantity of hyaluronidase remaining after using sodium salicylate.

CD—Quantity of hyaluronidase inactivated by 10  $\gamma$  of sodium salicylate.

total quantity of 34.7  $\gamma$  of enzyme. This difference must have been inactivated by the sodium salicylate added. From the concentration and volume of the inhibitors, the amount of enzyme inactivated by 1 mg can be calculated. This procedure was repeated for each one of the various inhibitors tested.

Before any inhibitor was used, certain criteria were established to eliminate invalidating factors. These were as follows:

1. Solutions of the inhibitor must be clear.
2. Alcohol solutions were diluted 1:10 with buffer pH 6.0 to ascertain whether mere dilution produced a precipitate.
3. Non-specific protein precipitating action of the inhibitors were tested by adding them to the acidified normal horse serum which was diluted with 3 parts of buffer pH 4.2.
4. Extensive studies of all pHs were carried out (Table I). Two buffers of pH 6.0 and 4.2 were checked periodically on a Beckman

TABLE I.  
 Comparative List of Hyaluronidase Inhibitors.

%	Inhibitor		pH of buffer (pH 6.0) + inhibitor	pH of mixture: buffer pH 6.0 + buffer pH 4.2 + inhibitor	$\gamma$ of hyaluronidase inactivated by 1 mg of inhibitor
1	m-hydroxybenzoic acid	alc	5.52	4.25	3.2
1	p-hydroxybenzoic acid	"	5.65	4.25	3.6
1	ethyl p-hydroxybenzoate	"	6.12	4.26	7.0
5	phenol	aq	6.02	4.23	7.5
5	resorcinol	alc	6.12	4.25	8.4
1	o-nitrophenol	"	6.06	4.25	19.7
1	guaiacol	aq	6.02	4.24	24.0
1	m-cresol	"	6.03	4.26	25.0
1	methyl p-hydroxybenzoate	alc	6.12	4.25	28.0
1	propyl p-hydroxybenzoate	"	6.12	4.25	31.0
1	4-aminosalicylic acid	"	5.68	4.26	31.7
1	o-cresol	"	6.12	4.26	37.5
1	dinitrophenol	"	5.72	4.25	39.6
1	p-chlorophenol	"	6.12	4.25	42.0
1	o-chlorophenol	"	6.13	4.25	44.2
1	picric acid	aq	5.68	4.26	46.4
1	butyl p-hydroxybenzoate	alc	6.12	4.25	93.0
0.1	hexylresorcinol	"	6.12	4.26	400.0
.025	diamyl phenol	"	6.12	4.27	600.0
.052	2,5 ditertiary butylhydroquinone	"	6.10	4.27	669.3
10	sodium salicylate	aq	6.02	4.26	1.5
5	sodium gentisate	"	5.85	4.27	0.7

pH meter. The readings were between 5.95 and 6.02 for the former and 4.15 and 4.20 for the latter.

To ascertain the pH of the solution containing buffer pH 6.0 and the same proportion of inhibitor as in the studies, *i.e.* 1:0.1, a thorough investigation was undertaken with each inhibitor (Table I) and the values obtained varied between 5.52 and 6.13. It is important also to note that there is no direct relationship between the variations in pH and the potency of drug inhibition and that the activity of hyaluronidase is maximal within these two extremes.

When the two buffers (6.0 and 4.2) were mixed in the same proportion as in the determinations, *i.e.* 1:3, the average pH value of the mixture was  $4.25 \pm 0.02$ . The effect of each inhibitor on this value was then studied by adding 1:10 of the inhibitor to the mixed buffers. As can be observed, the values are between 4.22 and 4.27 and well within the limits of experimental error under the conditions of this experiment. Thus, the influence of the inhibitor to displace the pH of the buffering system was eliminated and the action on the acidified horse serum was a variable of the enzyme factor alone; non-

specific action of the inhibitors was eliminated.

All data are tabulated in terms of gammas of hyaluronidase inactivated by one milligram of each compound. Since ethyl alcohol (95%), in the volume used, had no effect on the production of turbidity, it was used as a solvent for most of the drugs. In all other cases aqueous solutions were used.

*Discussion.* At this time we may speculate on the clinical importance of these findings. If an etiological relationship can be presupposed between hyaluronidase and acute rheumatic fever, the need for massive doses of sodium salicylate should be explained by our findings, since this drug is a weak inhibitor of hyaluronidase. As a matter of fact, Coburn(14,15) advocated plasma levels of at least 25 mg per 100 cc to secure prompt and progressive subsidence of the inflammation. This concentration usually makes the patient toxic with tinnitus, nausea, etc. Recently, Fulton *et al.*(16) reported that concentrations of sodium salicylate up to 100 mg % failed to inhibit hyaluronidase in

14. Coburn, A. F., and Kopp, J. *Exp. Med.*, 1943, v77, 173.

15. Coburn, A. F., *Bull. Johns Hopkins Hosp.*, 1943, v73, 435.

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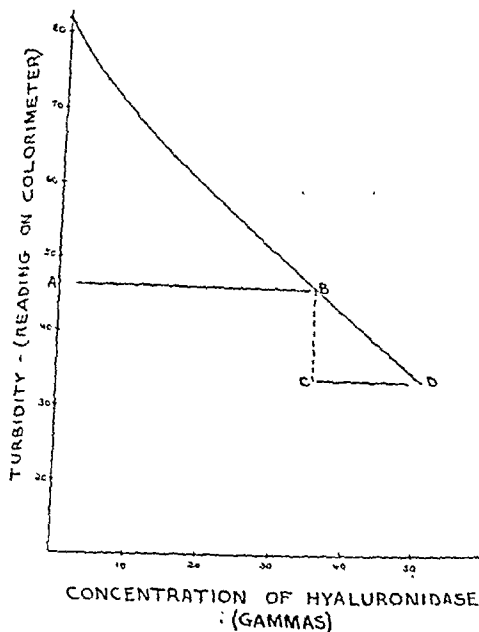


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TABLE I.  
Occurrence of Lymphomatosis in Bagg Albino Mice Receiving Threshold Doses of Lymphomogenic Agents Either Singly or in Combination.

Agent	Total mice	Mice over 250 days	Total lymphoma	Mediastinal lymphoma	Age lymphoma (in days)	Dead and negative	No. living*
200r†	105	70	3	1	365	10	92
EDP‡	59	30	2	2	350	7	50
200r + EDP	70	36	15	12	283	13	42
Controls	101	101	32	1	725	69	0
18MC§	108	76	1	0	490	23	84
200r + 18MC	109	75	3	2	365	18	89
EDP + 18MC	58	19	3	1	285	5	50

\* The oldest EDP-treated test animals (alone or in combination) are 500 days of age. The oldest MC-treated mice are 700 days of age.

† 20 r of x-rays in one exposure.

‡ Estradiol dipropionate—5  $\mu$ g weekly for 14 weeks. This estrogen was supplied by Ciba Pharmaceutical Products through the courtesy of F. E. Houghton.

§ Methylcholanthrene—18 skin paintings with 0.25% solution in benzene.

TABLE II.  
Occurrence of Lymphomatosis in CBA Mice Receiving Threshold Doses of Lymphomogenic Agents Either Singly or in Combination.

Agent	Total mice	Mice over 250 days	Total lymphoma	Mediastinal lymphoma	Age lymphoma (in days)	Dead and negative	No. living*
200r	148	83	6	1	410	8	134
EDP	57	42	2	1	294	2	53
200r + EDP	55	33	11	11	293	2	42
Controls	63	63	20	0	608	43	0
18MC	126	103	1	0	405	20	105
200r + 18MC	118	79	8	0	445	17	93
EDP + 18MC	54	16	0	0	—	6	45

\* The oldest EDP-treated test animals (alone or in combination) are 500 days of age. The oldest MC-treated mice are 700 days of age.

represent a threshold dose for this agent. Two groups of mice, one in the Bagg albino and the other in the CBA strain, each received a single dose of 200 r of x-rays at ages ranging from one to 70 days. Two groups from the same stocks received 14 weekly injections of 5  $\mu$ g of estradiol dipropionate in peanut oil beginning at 42 days of age. In a third treatment series 2 groups received 200 r at 42 days followed by the 14 weekly injections.

Although 32% of untreated Bagg albino mice developed lymphomatous disease, the average age of appearance was 725 days, with the earliest spontaneous case being observed at 490 days. Only one of the 32 cases was a mediastinal (thymic) lymphosarcoma (Table I). In the CBA strain also (Table II) the average age of appearance of leukemia was late, 608 days, with only 2 of 20 spontaneous cases appearing before 515 days. None of these spontaneous cases was a mediastinal

lymphosarcoma. Thus, out of a total of 52 spontaneous cases of lymphomatosis in these 2 stocks of mice only one was a mediastinal lymphosarcoma. The low incidence of mediastinal lymphosarcoma can be correlated with a delayed appearance of systemic lymphomatosis (leukemia). Where spontaneous leukemia appears relatively early, as in the F strain, the incidence of mediastinal lymphosarcoma is high.(7) Microscopic study of early mediastinal lymphosarcoma indicates that in the mouse the site of origin is within the thymus itself.(8) Thymectomy has decreased the spontaneous incidence.(9)

When threshold leukemogenic doses of x-

7. Kirschbaum, A., Strong, L. C., and Gardner, W. U., *Proc. Soc. Exp. Biol. and Med.*, 1940, **45**, 287.

8. Kaplan, H. S., *Cancer Research*, 1947, **7**, 141.

9. MacEndy, D. P., Boon, M. C., and Furth, J., *Cancer Research*, 1944, **4**, 377.

*vitro*. In order to obtain only a slight effect of this enzyme in our experiments, we were forced to raise the salicylate concentration ten times higher than Fulton by using 10 mg of sodium salicylate per cc which is beyond the range of therapeutic dosage. In view of recent clinical investigations,(17) there is a possibility that the modus operandi of salicylates is a stimulation of the adrenal glands liberating a hormone which relieves the rheumatic manifestations. Sodium gentisate, which is a metabolic product of salicylate is even weaker. In the study of 4-amino salicylic acid (PAS), the anti-hyaluronidase activity approaches that of ortho cresol with, however, a higher therapeutic index.

Of the utmost importance is the fact that

16. Fulton, J. K., Marcus, S., and Robinson, W. D., *Proc. Soc. Exp. Biol. and Med.*, 1948, v69, 258.

17. Hench, Kendall, Slocumb, and Polley, *Proc. Staff Meetings of the Mayo Clinic*, 1949, v24, 181.

neither benzoic acid nor para aminobenzoic acid inhibit hyaluronidase, whereas their hydroxyl derivatives do.

*Summary.* The anti-hyaluronidase activity of phenol substitution products increases markedly with the length of the carbonic side chain. Salts of aromatic acids, like sodium salicylate, have an activity of a lower order as compared with that of phenols with long carbonic side chain. A moderate increase in activity is also obtained by substituting chlorine, nitro or amino groups.

As the data indicate, there exists in general, a parallel course of the inhibitory power of the substances and their phenol coefficient.

We wish to thank S. Joffe for valuable laboratory assistance.

Both the enzyme and substrate used in these experiments were obtained from the Sehering Corporation.

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### Synergistic Action of Estrogenic Hormone and X-rays in Inducing Thymic Lymphosarcoma of Mice.\* (17522)

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Lymphocytic neoplastic disease was induced by estrogens(1) or x-rays(2) in mice of certain genetic constitution when these agents were given in appropriate doses. The carcinogenic hydrocarbons are another group of lymphoma-inducing agents.(3) Synergistic or additive lymphomogenic effects of x-rays and the carcinogenic hydrocarbon, methylcholanthrene, were demonstrable(4) providing

lymphoid tissue of the test animals was susceptible to the neoplasia-inciting action of each of the agents used independently.(5) In the present experiment the synergistic action of x-rays and estrogens was investigated. It had previously been demonstrated that the CBA and Bagg albino stocks of mice are susceptible to the induction of lymphomas by the action of either estrogens or x-rays.(6) A single dose of 200 r of x-rays is a minimum inducing dose; weekly injections of 5 micrograms of estradiol dipropionate for 14 weeks

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## Liver Glycogen Response to Adrenal Cortical Extract of Diabetic and Non-Diabetic Rats.\* (17523)

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This paper is a report of the liver glycogen response of alloxan-diabetic rats, following adrenalectomy, to adrenal cortical extract. It was of interest to determine to what extent liver glycogen could be increased by adrenal cortical hormones in the absence of an adequate insulin supply.

It has been previously reported(1-3) and confirmed in this paper (Table I), that the livers of alloxan-diabetic rats contain more glycogen than normal rat livers following a 24-hour fast. When fed (4-6) or comatose diabetic (2,7) animals have been used, a higher level of glycogen in the diabetic animal was not found. This difference in the liver glycogen levels of the fasted normal and diabetic animals did not complicate the experiment, however, since all animals were adrenalectomized 3 days prior to injection of adrenal cortical extract (ACE). The liver glycogen of the adrenalectomized diabetic rat ( $0.012 \pm 0.002$  mg/100 g liver) is of the same low order of magnitude as the adrenalectomized normal rat ( $0.016 \pm 0.003$  mg/100 g liver, Table II).

**Experimental.** Sprague-Dawley rats, male and female, weighing approximately 100 g

(range, 80-110 g) were given alloxan (generally 130 mg/kg) subcutaneously following a 24 hour fast. For the following 24 hours, 5% glucose was substituted for drinking water. Nonfasting blood sugars were determined the following week by a modification of Folin's micromethod.(8) The animals were adrenalectomized 6-10 days later, and 3 days after adrenalectomy liver glycogen was determined by the method used by Dorfman *et al.*(9) Upjohn's lipo-adrenal extract (1 cc  $\approx$  2 mg 11-dehydro-17-OH-corticosterone) was used. In the first experiment (Table II) this was diluted 1:1 with mazola oil. Following a 24 hour fast, each of the injected animals received 4 subcutaneous doses of  $\frac{1}{4}$  cc at 2 hour intervals, and all animals were sacrificed 2 hours after the last injection. The animals were divided into 4 groups: (1) adrenalectomized, (2) adrenalectomized-diabetic, (3) adrenalectomized plus ACE, (4) adrenalectomized-diabetic plus ACE; the results are tabulated in Table II.

The results indicate that there is no statistically significant difference between the liver glycogen response to ACE of diabetic and non-diabetic rats following adrenalectomy. If the diabetic animal has reduced liver hexokinase activity or other defect causing reduced phosphorylation of sugar, it is possible that glycogen is formed largely from carbon fragments smaller than hexose; this is in agreement with the interpretation by Stetten(10) of the defects in alloxan diabetes.

**Summary.** Liver glycogen of alloxan-diabetic rats was 8 times as great as in normal rats. Both groups were fasted 24 hours. The liver

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TABLE III.

Occurrence of Lymphomatosis in dba Mice Receiving Threshold Doses of Methylcholanthrene and X-rays Either Singly or in Combination.

Agent	Total mice	Mice over 250 days	Total lymphoma	Mediastinal lymphoma	Age lymphoma (in days)	Dead and negative	No. living*
200r	78	38	0	0	—	13	69
18MC	105	94	21	2	325	47	37
200r + 18MC	97	78	29	14	320	20	48
Controls	117	117	19	3	350	95	0

\* The oldest living mice are 700 days of age.

rays and estradiol dipropionate were given simultaneously to Bagg albino or CBA mice, synergistic effects were observed. Although a large per cent of the mice are still living (Tables I and II), of the 39 mice on combined treatment which have died 25 had lymphomatosis, and in 22 cases the disease was mediastinal lymphosarcoma.

Methylcholanthrene is not leukemogenic for these stocks.(6) When 18 skin paintings of 0.25% solution in benzene were combined with either 200 r of x-rays or 5 micrograms of estradiol dipropionate weekly for 14 weeks, the incidence of lymphomatosis was no greater than when either of the latter agents was administered alone (Tables I and II). In the dba stock, in which methylcholanthrene is leukemogenic,(3,6) the incidence of lymphomatosis was increased (Table III) when x-rays and the carcinogen were given simultaneously. The increased incidence can be accounted for solely by the greater number of mediastinal lymphosarcomas, the incidence of generalized lymphomatosis being the same whether methylcholanthrene was given alone or combined with x-radiation.

These experiments indicate that the thymus of young mice of certain strains contains lymphoid tissue which is very sensitive to the lymphomogenic action of certain agents. Al-

though systemic lymphomatosis (leukemia) developed at an advanced age in the Bagg albino and CBA stocks, spontaneous thymic lymphosarcoma appeared only once in a population of 164 controls. Thus lymphocytic neoplastic transformation within the thymus represents a disease which appeared spontaneously only occasionally although it could be readily induced.

A total of 70  $\mu$ g of estradiol dipropionate acted as a synergist in the induction of mediastinal lymphosarcoma. This amount of estrogen, when given from 42 to 140 days of age, was not sufficient to inhibit the ability of test animals to resume reproductive activity. In the development of spontaneous leukemia multiple factors may be operating synergistically so that significant causative ones may be obscured.

*Summary.* Synergistic lymphomogenic effects were obtained when estrogenic hormone and x-rays were administered simultaneously to mice of the Bagg albino and CBA stocks. Thymic lymphoid tissue appeared to be more sensitive than other lymphoid tissue. This was also true when x-rays and methylcholanthrene acted synergistically in the dba stock.

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considerable; we were thus led to a study of a possible *in vitro* reaction that might be applicable for the identification of the virus, namely, hemagglutination or complement fixation. The present report concerns itself with the results of studies of the two reactions employing 2 of the Albany strains, Type I (T.T.) and Type II (Fleetwood), generously presented to us by Dr. Dalldorf.

**Hemagglutination Tests.** Agglutination tests were carried out in a manner already described,<sup>(5)</sup> except that the saline solution used for dilution of materials was buffered with 0.02M, instead of 0.05M phosphate. Human group O, sheep, chick, and guinea pig RBC were employed and the two different strains, Type I and II, of the Cocksackie virus. In addition, two forms of each strain were studied; suspensions of infected brain and of limbs, derived from infant mice which had reacted after their injection intraperitoneally of either active brain or limb virus.<sup>(6)</sup> The tubes containing materials were held at 5, 23, and 37°C and read at intervals up to 2 hours. As an indicator of the possibility of the materials yielding positive reactions, a test was set up with sheep cells and MM virus; definite agglutination was seen in 1:160 dilution of the virus after 1 hour at 5°C.<sup>(5)</sup> The results of all the hemagglutination tests with the 2 strains of Cocksackie virus were, however, negative. It should be stressed that suspensions of normal mouse brain, especially of fresh brain, if not properly centrifuged, show agglutination of sheep RBC, the degree of hemagglutination depending on the particles in the supernate.

**Complement-fixation tests.** Complement-fixation tests have been carried out with the same Albany strains using mouse immune serum and antigens derived from infected brain or limb tissue of suckling mice, Swiss-W strain.<sup>‡</sup>

**Immune Serum.** Antisera were prepared in 3- to 4-month-old Swiss-W mice by giving

each of them, intraperitoneally, 0.5 ml of mouse-brain virus,  $10^{-1}$  dilution, on the 1st and 5th day; they were bled on the 15th day. These animals were then injected intraperitoneally with limb virus, each with 0.5 ml of  $10^{-1}$  dilution, on the 19th and 25th day; they were bled again on the 35th day. In all 20 mice were so treated with each type of virus.

**Antigen for test.** Antigens for complement-fixation tests were prepared following a method previously described (acetone-ether extracted antigens).<sup>(7)</sup> In the present study, 2- to 11-day-old Swiss mice were given .05 ml of  $10^{-1}$  suspension of infected brain or limb tissue intraperitoneally. When definitely ill,<sup>‡</sup> usually on the 3rd day, brain and limb tissue, the skin being removed from the latter, was collected separately and prepared<sup>(7)</sup> as antigens. It should be stated here that the titer of virus in limb tissue derived from 11-day-old mice was greater than  $10^{-7.5}$ , confirming the observations of Gifford and Dalldorf.<sup>(2)</sup> Extraction of the limb tissue with acetone-ether was found to be more satisfactory than the method of suspension in saline solution followed by centrifugation at 12,000 rpm. For control material, brain and limb tissue was collected from apparently normal Swiss mice of the same age. The complement-fixation test was performed in the manner previously described.<sup>(8)</sup>

**Experimental.** Table I illustrates one of the tests. A specific fixation was obtained with both types of Cocksackie virus, used as limb-tissue antigen, without any demonstrable sign of cross reaction. Moreover, antigens made up of brain-tissue virus were not effective. Indeed, following the injection of brain virus into mice, their sera were either negative or of a low titer; whereas after immunization with limb-tissue virus a high titer of fixing antibody was obtained. It would appear that the limb-tissue virus is a highly potent antigen. Finally, no reaction took place between Cox-

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TABLE I.  
Liver Glycogen in Normal and Diabetic Rats (Fasted 24 Hr).

Status	No. of animals	Liver glycogen, mg/100 g liver	P	Blood sugar, mg% (mean and range)
Diabetic	11	.65 $\pm$ .14	<.01	658 (403-1100)
Normal	13	.08 $\pm$ .03		
Non-diabetic (received alloxan)	6	.14 $\pm$ .07		

TABLE II.  
Liver Glycogen Following Administration of Adrenal Cortical Extract to Diabetic and Non-diabetic Rats. (All animals adrenalectomized and fasted 32 hr).

Status	No. of animals	Treatment	Liver glycogen, mg/100 g liver	P	Blood sugar,† mg% (mean and range)
Diabetic	14	0	.012 $\pm$ .002	>.2	410 (264-620)
Non-diabetic	17	0	.016 $\pm$ .003		
Diabetic	7	ACE*	.44 $\pm$ .08	>.9	380 (280-645)
Non-diabetic	9	ACE*	.43 $\pm$ .10		
Diabetic	9	ACE	1.67 $\pm$ .16	>.2	397 (296-556)
Non-diabetic	10	ACE	1.97 $\pm$ .16		

\* Diluted 1:1. † Prior to adrenalectomy.

glycogen response to adrenal cortical extract of diabetic rats was found not to be significantly different from the response of non-

diabetic rats. Both groups were adrenalectomized and fasted 32 hours.

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## Hemagglutination and Complement Fixation with Type I and II Albany Strains of Coxsackie Virus.\* (17524)

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Dalldorf and Sickles(1) isolated a virus from the feces of patients having symptoms resembling those of abortive or even paralytic poliomyelitis. This active agent, characterized by pathogenicity for suckling but not for adult mice, was later named Coxsackie virus.(2) Melnick, Shaw, and Curnen(3)

recovered several similar viruses some of which they found to be distinct by the neutralization test. Dalldorf(4) also determined such specificity for his strains, here designated as Type I and Type II Albany strains. The difficulties of working with infant mice are

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\* With the technical assistance of Joan Fitzgerald and Barbara Quinn.

† Major, V.C., U. S. Army.

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TABLE I.

Effect of Administration of Epinephrine and Refrigeration on Adrenal Ascorbic Acid Content.

Age, days	ascorbic acid, % of control value	
	Epinephrine	Refrigeration
2	93	
4	98	95
6	91	101
7		105
8	69	
10	66	
11		105
14		107
16		83
25		81
Adult	62	

TABLE II.

Effect of 2.5 Mg ACTH on Adrenal Ascorbic Acid Content.

Age, days	ascorbic acid, % of control value
4	66
6	64

the animals had reached 8 days of age. (Table I). Exposure of animals to refrigeration (5°C) for 2½ hours similarly did not result in a decline in the ascorbic content until the 16th day of life. With this stress the fall in ascorbic acid was 19% or approximately one-half of that with epinephrine (Table II).

Since very young rats do not respond to stress, it was necessary to further localize the

point of insensitivity, *e.g.*, the pituitary or adrenal. Therefore, rats of 4 and 6 days of age were injected intraperitoneally with 2.5 mg† ACTH and killed 80-120 minutes later. Ascorbic acid analysis of the adrenals revealed a 34-36% decline in the vitamin C content under these conditions (Table II).

It would appear from these results that the fall in adrenal ascorbic acid in the newborn rat does not occur as a result of exposure to stress at it does in the adult animal. Since the adrenals are capable of responding to the administration of ACTH at this age, it is probable that the adenohipophysis is the organ which is not capable of responding to stress by secreting ACTH until the animal has reached a certain state of maturation.

**Summary.** The administration of epinephrine to the infant rat does not result in a fall in adrenal ascorbic acid until the animal reaches the eighth day of life. Under the conditions of the experiment, adrenal ascorbic acid does not decline as a result of refrigeration until the 16th day of life. The administration of ACTH, on the other hand, causes adrenal ascorbic acid depletion on the 4th and 6th day of life.

† Armour ACTH, kindly supplied by Dr. John R. Mote, Medical Director of Armour Laboratories.

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## Labeling of Rat Hemoglobin with Radioactive Iron.\* (17526)

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In the studies of the biological aspects of iron metabolism, radioactive iron serves as a suitable means of following, *in vivo*, transformations of forms of iron. Especially valuable is its use in tagging hemoglobin molecules so that, under certain conditions, hemoglobin iron, hemoglobin molecules or red cells may be identified as they undergo

certain chemical or physical changes. The current methods(1-7) used for incorporating

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\* Aided by a grant from the New Orleans Academy of Sciences to H. S. Mayerson and John K. Hampton, Jr.

TABLE I.  
Showing Complement-fixation Tests with Albany Strains of Coxsackie Virus.

Antigen	Serum					Western equine
	Type I brain	Type I limb	Type II brain	Type II limb	Normal	
Type I limb	0	1/16	0	0	0	0
Type I brain	0	0	0	0	0	0
Type II limb	0	0	1/2	1/32	0	0
Normal limb	0	0	0	0	0	0
Western equine brain	0	0	0	0	0	1/64

Fractions represent the highest dilution of serum showing 2+ or better complement fixation; in the test, serum was diluted 2-fold beginning with 1:2 dilution.

sackie-virus sera or antigens and unrelated virus or apparently normal materials.

*Summary.* Two Albany strains of Coxsackie virus, Type I (T.T.) and Type II (Fleetwood) were found to be negative for hemagglutination when human O, sheep, chick, and guinea pig erythrocytes were used. On the other hand, the viruses showed distinct

positive specific complement fixation without any cross reaction between them. Antigens prepared from infected brain tissue were inactive; those from infected limb tissue were highly active. Tests with human sera and limb-tissue antigens are left for further study.

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## The Pituitary-Adrenal Relationship in the Infant Rat. (17525)

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The administration of adrenocorticotrophic hormone (ACTH) to the rat or guinea pig results in an almost immediate fall in adrenal ascorbic acid content and a more gradual decline in adrenal cholesterol.(1,2) This has been correlated with evidence of adrenal cortical secretion.(3) It had been previously demonstrated by Selye(4) that exposure to stress or the injection of noxious substances causes the secretion of ACTH by the adeno-hypophysis of the intact animal. Exposure

of animals to these same conditions causes a depletion of adrenal ascorbic acid in the intact animal but not in the hypophysectomized animal.(5) The purpose of this investigation was to determine whether the newborn rat could respond to stress and the administration of ACTH in a manner similar to that of the adult.

Infant rats at 2, 4, 6, 8 and 10 days of age were injected with 0.04-0.05 mg of aqueous epinephrine and the adrenals removed one to two hours later. In all, 170 rats from 27 litters were used in this study. Adrenal ascorbic acid was determined by the method of Roe and Kuether.(6) It was found that no decrease in adrenal ascorbic acid occurred until

\* Present address, Departments of Medicine and of Obstetrics and Gynecology, College of Physicians and Surgeons, Columbia University.

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point of insensitivity, *e.g.*, the pituitary or adrenal. Therefore, rats of 4 and 6 days of age were injected intraperitoneally with 2.5 mg† ACTH and killed 80-120 minutes later. Ascorbic acid analysis of the adrenals revealed a 34-36% decline in the vitamin C content under these conditions (Table II).

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**Summary.** The administration of epinephrine to the infant rat does not result in a fall in adrenal ascorbic acid until the animal reaches the eighth day of life. Under the conditions of the experiment, adrenal ascorbic acid does not decline as a result of refrigeration until the 16th day of life. The administration of ACTH, on the other hand, causes adrenal ascorbic acid depletion on the 4th and 6th day of life.

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## Labeling of Rat Hemoglobin with Radioactive Iron.\* (17526)

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From the Department of Physiology, Tulane University, School of Medicine.

In the studies of the biological aspects of iron metabolism, radioactive iron serves as a suitable means of following, *in vivo*, transformations of forms of iron. Especially valuable is its use in tagging hemoglobin molecules so that, under certain conditions, hemoglobin iron, hemoglobin molecules or red cells may be identified as they undergo

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radioactive iron as a heme constituent in hemoglobin often present major difficulties. Most iron compounds have a high toxicity and low stability which limits their use. The low specific activity and soft beta radiation of the available radioactive iron make it necessary to obtain a high percentage of tagging for most studies. Finally, since synthesis can be accomplished only in donor animals, preliminary production of anemic animals with drastically reduced iron stores is a time consuming and, at times, unpredictable procedure involving many serial bleedings and careful diet considerations.

In the course of iron studies in our laboratory, we have developed a procedure which we feel will provide an easier and, perhaps, a more satisfactory tagging of hemoglobin with radioactive iron than can be achieved with the methods which have been used. The iron used contained a mixture of Fe-55 and Fe-59 prepared by pile bombardment of enriched Fe-58. Concentrated hydrochloric acid, in amount approximately twice the molarity required for quantitative reaction with the iron sample was diluted with 3 parts of distilled water and poured over the powdered iron in a large flask. The reaction was allowed to continue, with heat supplied as the reaction slowed, until it was complete as indicated by a clear green solution without significant insoluble residue. This solution of ferrous chloride in excess hydrochloric acid was filtered, a sample analyzed by the Wong thiocyanate method(8) for iron and the remainder stored in a glass stoppered stock bottle. Working solutions were prepared by withdrawing from the stock the amount of iron desired and placing it in an appropriate volumetric flask. The HCl and water were removed by evacuation and heat. The green ferrous chloride powder was dissolved in 0.1 N lactic acid and the flask filled to the mark with this acid. The concentrations of ferrous chloride and lactic acid were considered in terms of volume which would be

desirable for injection, the amount and concentration the animal could tolerate and the relative molarities of the iron and lactate which would provide a pH and reducing environment for the iron suitable for prolonged storage at room temperature. These conditions were met by preparing a 400 mg % Fe solution of ferrous chloride in 0.1 N lactic acid. The solution was sterilized by Seitz filtration and stored in a sterile rubber-capped bottle at room temperature. Such working solutions show no signs of deterioration after more than two months of storage.

Adult male and female white rats were used. Their weights ranged between 225 and 330 g. By cutting the tails and suspending them in graduated centrifuge tubes containing warm sodium citrate, the animals were bled about 1.5% of their body weight at 2-3 day intervals for 11 bleedings. Their hematocrit values dropped to an average of about 25%. At this point, iron injections were begun and during the course of the injections four more bleedings were performed. Also the usual Rockland Rat diet was supplemented by milk at this point. Response by weight gain, increased appetite and general vigor was apparent within one week. At no time were they maintained on a diet considered low in iron.

The working lactic acid-iron solution was injected into rats intraperitoneally in doses of 0.25 cc per 100 g of body weight, which provided 1 mg of iron per 100 g of body weight. These injections were given daily for the first half and every other day for the remainder of the series. A total of 14 injections were given, of which the third and tenth injections were at one-half dose. The total amount of iron thus administered was 13 mg/100 g of body weight. This is notably in excess of the animal's iron requirement.

The low toxicity of the injections is evident in animals in good health. Of 12 rats, 4 deaths occurred after iron injections were begun. One of the deaths occurred as a result of the 12th bleeding. The other 3 followed the 2nd, 7th and 9th injection of lactic acid-iron solution. Although peritonitis could not be excluded because of the irritation inflammation produced by the acid, death followed not

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TABLE I.

Percentage Tag of Hemoglobin with Radioactive Iron. The stock radioactive iron injected gave 3,300 counts/min./mg of iron. The values represent no duplicates since the blood was collected by exsanguination and pooled for each lot.

Sample	No. animals	Days since last inj.	Counts/min./mg Fe	% tag
4	3	-2	957	29.0
5	2	1	850	25.8
6	2	3	978	29.7
7	1	19	988	29.8

earlier than 12-24 hours after the injection.

Radioactivity determinations were made by counting desiccated protein-free filtrates and chemical iron was determined on such filtrates by the method of Wong.(8) Counts were sufficiently high under the geometric and sensitivity conditions employed to allow accurate comparison of samples.

In Table I are recorded the percentages of tagging achieved by our method. To be noted is the reasonable consistency of the values, each of which were obtained from a separate group of animals. Once bleedings were stopped, then no appreciable increase in tagging seemed evident over a 3-week period. After iron injections were begun, but before bleedings were stopped, a definite increase in tagging was apparent at weekly intervals.

Although we have demonstrated that a 25-30% tag may be accomplished in 48 days, in rats, we feel that the institution of milk supplement to the diet earlier in the experiment would have eliminated some of the emaciation. Furthermore, the distress ob-

served in the animals with the first few injections might have been eliminated. We also feel that satisfactory tagging could be accomplished with smaller doses, thus minimizing adhesions and iron staining of the viscera which was noted at autopsy. It is believed that if a greater percentage of tagging was desired it could be accomplished by continuing the serial bleeding and iron injections for a longer period of time.

*Conclusions and summary.* The tagging of hemoglobin in the red cells of rats by the method described is efficient. The iron compound, ferrous chloride, is of low toxicity, the lactic acid in which it was dissolved provides a satisfactory stabilizing environment and the solution is well tolerated when injected. The data show a consistency in the percentage of tagging as evidence of the reproducibility of the experiment. In addition to the ease with which the materials are prepared, the short duration of the experiment is a desirable feature.

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## Effects of Hemorrhagic Shock upon the Electroencephalogram. (17527)

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The central nervous system is extremely sensitive to reduction of its oxygen supply, particularly the small pyramidal cells of the cerebral cortex.(1) In view of the general acceptance of this fact, it is surprising that

studies have not been made of the effects of anemic anoxia produced by hemorrhage upon the electrical activity of the cerebral cortex as determined by the electroencephalograph.

*Materials and methods.* Rabbits weighing 5 to 11 lb were used in these experiments. General anesthesia was not used. Curare (In-

1. Gomez, L., and Pike, F. H., *J. Exp. Med.*, 1909, v11, 257.



tocostrin) was employed to abolish skeletal muscular movements which affect the electroencephalogram. Curare was used in a dose of 0.7 unit per lb. of body weight. The 4 electrodes of the electroencephalograph were placed in similar positions on the heads of the animals throughout these studies. The right femoral artery was exposed under local anesthesia (5 cc of 1% novocaine solution), cannulated and attached to a blood pressure recording manometer. Shock was produced by graded hemorrhage. The blood which was withdrawn was prevented from clotting by the addition of heparin solution. The heparinized blood was returned to the animals in many of the experiments.

**Results.** In control runs before curare was given, the electroencephalograms revealed a basic frequency of 6 to 7 cycles per second with an amplitude averaging about 75 microvolts. Numerous variations in the amplitude and frequency of the waves occurred through the record. There were short flurries of low voltage and of 20 to 30 cycle activity, and occasional sharp waves and spikes of 100 microvolts were observed. Following injection of curare, the rapid activity disappeared leaving a fairly symmetrical rolling type of wave, varying in frequency between 4 to 7 cycles per second and having an average amplitude of 50 microvolts.

Moderate to deep shock produced by bleeding resulted in slowing of the wave frequency to an average of 2 to 4 cycles per second. At the same time the amplitude of the waves increased to 150 to 200 microvolts and in some animals reached 400 microvolts. This was the basic pattern. There were, however, many irregular waves, sharp waves, rolling waves and occasional dicotic or notched waves. During recovery from shock the slow waves with high amplitude were replaced intermittently by rapid waves (10 cycles) of lower amplitude (100 microvolts).

If shock were progressive and transfusions were not given, the slow rolling waves persisted but their amplitude decreased. The voltage diminished and finally the wave pattern disappeared corresponding with the death of the animal. In those animals which survived without transfusion (reversible shock) the

basic pattern of slow rolling waves of increased amplitude was maintained until the conclusion of the experiment. In shock reversible by blood transfusion the wave pattern returned (sometimes within 10 minutes) to the type present before bleeding. The blood pressure returned to normal before the electroencephalogram. In shock irreversible by blood transfusion, the waves decreased in amplitude and frequency despite the administration of blood. It was noted in these experiments that the response of the electroencephalogram was a better prognostic criterion than was the response of the blood pressure. A persistent decrease in the frequency and amplitude of the waves was an ominous sign even though the blood pressure rose following blood transfusion. The longer the duration of shock, the more difficult was it to restore the electroencephalogram to normal with blood transfusions.

**Discussion.** The mechanism of production of the electroencephalographic changes in hemorrhagic shock may be briefly considered. Slow waves of increased amplitude have been observed in hypotension due to carotid sinus syncope,(2) insulin shock,(3) in anoxemia.(4) and after ligation of the carotid arteries for an intracranial saccular aneurysm.(5) The effect of low blood pressure due to stimulation of the vagus in depressing cortical electrical activity has been observed to be almost identical with that due to anoxemia.(6) It is suggested, therefore, that the electroencephalographic changes in hemorrhagic shock are due, in part at least, to cerebral anoxia.

**Summary and conclusions.** Hemorrhagic shock in rabbits produces definite changes in the electroencephalogram. These are characterized basically by the appearance of slow

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6. Bailey, P., and Bremer, F., *J. Neurophysiol.*, 1938, v1, 403.

waves of increased amplitude. In shock irreversible to transfusion there occurs a progressive decrease in cortical electrical activity which is shown on the electroencephalogram as a continued reduction in the frequency and voltage (amplitude) of the wave pattern. The effects on the electroencephalogram of blood

transfusion in reversible and irreversible hemorrhagic shock are discussed. It is tentatively suggested that the electroencephalographic response to blood transfusions in shock may be used as a means of differentiating the reversible and irreversible forms.

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### Vitamin B<sub>12b</sub>: Some Properties and its Therapeutic Use.\* (17528)

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The preparation of two red fractions from liver extract, (1) both clinically active in pernicious anemia, was reported by Smith who suggested that one fraction might arise from the other by proteolysis. In later publications (2,3) it was reported that paper chromatography enabled 2 red factors to be recognized in liver extract. Both of these were

active for *L. lactis* Dorner. In our investigations it was found that concentrated liver extract yielded a pink fraction by chromatography with silicic acid. Upon purification, this fraction yielded a red pigment with an absorption spectrum differing from that of vitamin B<sub>12</sub>. An apparently similar fraction was obtained from cultures of *Streptomyces aureo-*

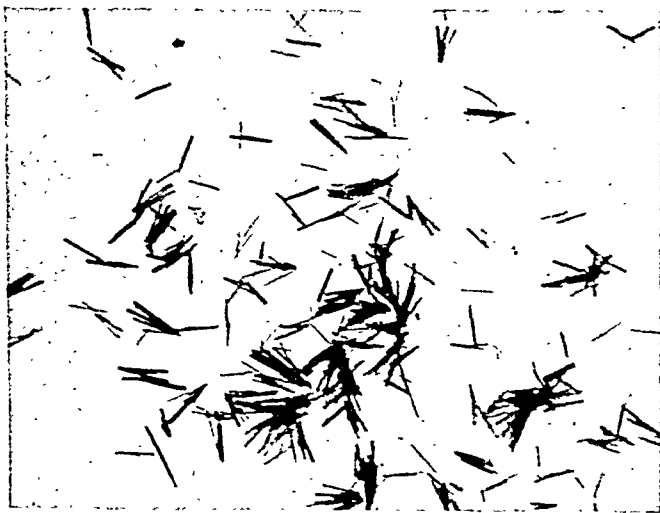


FIG. 1.  
Crystals of vitamin B<sub>12b</sub> ×425.

\* Paper presented at the Eastern Section of the American Federation for Clinical Research at Boston, Mass., on Dec. 3, 1940.

The expenses of this investigation were defrayed by a Lederle grant.

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3. Cuthbertson, W. F. J., and Smith, E. L., *Proc. Biochem. Soc.*, January 22, 1948.

TABLE I.  
Vitamin B<sub>12b</sub>: Hematological Response in Patients with Addisonian Pernicious Anemia.

Case No.	1	2	3	4	5
Vit. B <sub>12b</sub> , γ, I.M. daily	1	1	1	1.5	2
Initial RBC, mil/cmm	1.90	2.60	1.74	1.75	1.47
Day of reticulocyte peak	8	8	9	7	9
Max. reticulocytes, %	11.4	14.0	18.0	8.3	15.1
Expected max. retic., %	18	5	18	18	24
RBC, mil/cmm, at 3 wk	3.06*	4.36	2.84	3.17	2.95
Expected RBC, at 3 wk	3.11*	3.43	3.19	3.29	3.17

\* At 2½ wk.

*faciens* and red crystals of a biologically active compound "vitamin B<sub>12b</sub>"(4) were obtained from this fraction. The crystals of vitamin B<sub>12b</sub> are shown in Fig. 1. In the present investigation crystalline vitamin B<sub>12b</sub> was prepared from cultures of *S. aureofaciens* by absorption on charcoal, precipitation with ammonium sulfate, chromatography upon silicic acid columns and crystallization from acetone-water mixtures. The vitamin was dissolved in 0.9% sodium chloride solution at pH 7.0. The solution was sterilized by filtration through a Seitz filter. The final concentration of vitamin B<sub>12b</sub> was adjusted to 10 μg per ml.

**Clinical Observations.** The solution of crystalline B<sub>12b</sub> containing 10 μg per ml was used for assay in 5 patients with Addisonian pernicious anemia. All patients had megaloblastic bone marrows and histamine-fast achlorhydria. The age range was 55 to 75 years. Vitamin B<sub>12b</sub> was administered by intramuscular injection daily for 21 days in the dosage schedule given in Table I. Hematologic response seemed to be optimal whether the amount given was 1, 1.5, or 2 micrograms. One patient had a higher reticulocyte peak than expected, one the same and three somewhat lower. The relative unreliability of the reticulocyte count as a quantitative index of the formation of red blood cells has been recently emphasized by several observers.(5,6) In contrast to the fluctuation of the reticulocyte peak in relation to that of the expected

standard, the erythrocyte regeneration was within maximal limits in all 5 patients at the end of 21 days of therapy. It thus seems that 1 to 2 μg of vitamin B<sub>12b</sub> is hemopoietically equivalent to 1 U.S.P. unit of liver extract and is then comparable to the potency of vitamin B<sub>12</sub>.(6,7)

The detailed hematologic data of Case 2 who showed the most dramatic response are presented in Fig. 1. Therapy resulted in the usual reversal of the megaloblastic marrows to normal normoblastic cellularity. The white blood cell counts rose from leukopenic to normal figures with a loss of hypersegmentation of the neutrophils. In the only patient (Case 3) who has been maintained longer than 3 weeks on vitamin B<sub>12b</sub>, the red cell count has risen to a nearly normal level at the end of 7 weeks.

Clinical improvement was also very satisfactory, beginning on the 3rd, 4th or 5th day of treatment, and was characterized by improved appetite and a sense of well-being. Severe glossitis in one patient completely subsided within 10 days. Neurological symptoms such as paresthesias disappeared in all cases. A patient who had advanced combined system disease had striking improvement within 3 weeks, including the reversal of a strongly positive Romberg's sign.

Oral administration of 5 μg of vitamin B<sub>12b</sub> daily for 10 days was undertaken with a 6th patient with Addisonian pernicious anemia, having an initial red cell count of 2,090,000 per cmm. There was no response, the reticulocytes varying from 0.4% to 1.4%. The patient was then given the same therapy with

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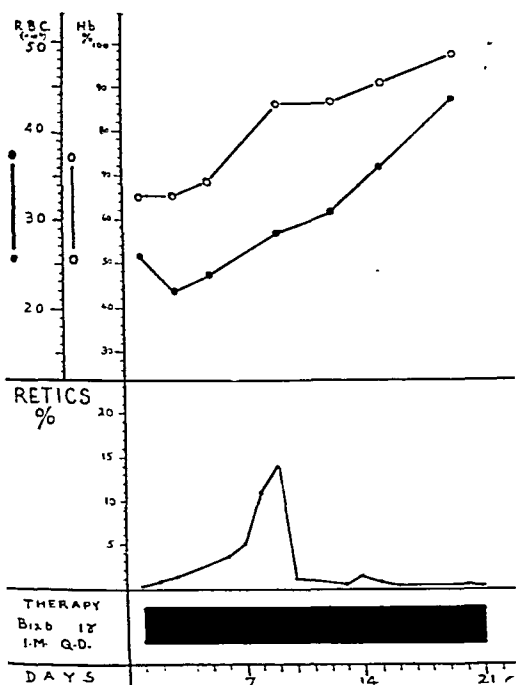


FIG. 2.

Hematological response of a patient with Addisonian pernicious anemia to vitamin B<sub>12</sub>. (Case 2).

the simultaneous addition of 150 ml of neutralized gastric juice daily for 12 days. The reticulocytes began to rise slowly on the 8th day to reach a peak of 3.4% on the 11th day. There was a concomitant red blood cell rise of 240,000 per cmm during this time, and slight but definite clinical improvement occurred. This is similar to the results obtained by Berk *et al.*(8) using vitamin B<sub>12</sub>.

We observed one patient with megaloblastic anemia of pregnancy who had failed to respond to 3 weeks of therapy with 150 U.S.P. units of refined liver extract intramuscularly, and also showed no hematologic response to 1 µg daily of vitamin B<sub>12b</sub> given intramuscularly in a 10 day period. The severe glossitis, unaffected by liver therapy, cleared completely on B<sub>12b</sub>, and later there was a maximal hematologic response to pteroylglutamic acid. Several similar vitamin B<sub>12</sub> refractory cases have been reported. The problem of different types of deficiencies occurring in megaloblastic anemias other than Addisonian pernicious anemia has been recently reviewed by Mueller *et al.*(9)

**Summary.** A pink fraction obtained from liver and from *Streptomyces aureofaciens* has been separated from vitamin B<sub>12</sub> by silicic acid chromatography and has been shown to have different ultraviolet and visible absorption spectra. This new substance, vitamin B<sub>12b</sub>, has been prepared in crystalline form and has been found to be effective parenterally in the treatment of patients with Addisonian pernicious anemia in amounts of 1 to 2 µg daily.

We are indebted to Mrs. Helen Jakubowski for performing the blood examination.

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## Sterols and Fatty Acids in the Nutrition of Entozoic Amoebae in Cultures.\* (17529)

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The many excellent media which have been

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suggested for culture of entozoic amoebae suffer from one serious deficiency when used for quantitative studies. Statistically satisfactory reproduction of results is the exception rather

TABLE I.  
Relative Growth of *E. histolytica* with Various Serum Fractions.

Transfer	Control (LSB)	Serum fractions, % of total								
		A			B			C		
		10	5	1	10	5	1	10	5	1
1	4+	3+	2+	+	4+	3+	3+	3+	2+	2+
2	4+	3+	3+	2+	4+	4+	2+	+	+	+
3	4+	2+	2+	+	4+	4+	3+	+	+	+
4	4+	+	+	+	3+	4+	3+	+	+	+
5	4+	+	+	—	3+	3+	4+	—	—	—
6	4+	—	—	—	3+	3+	3+	—	—	—
7	4+	—	—	—	4+	4+	4+	—	—	—
8	4+	—	—	—	4+	4+	4+	—	—	—
9	4+	0	0	0	3+	4+	4+	0	0	0
10	4+	0	0	0	4+	4+	3+	0	0	0
11	4+	0	0	0	4+	3+	3+	0	0	0

4+ = 40+/lpf.

3+ = 30-40/lpf.

2+ = 15-30/lpf.

1+ = 1-15/lpf.

+ = less than 1/lpf.

— = No detectable growth.

0 = Series discontinued.

than the rule. A fluid medium of constant composition is an absolute requirement. For several years we have used a modification of the Cleveland and Collier(1) and Chang(2) media in conjunction with physically purified rice starch. The medium (LSB) and method of preparing the starch have been described in a previous paper.(3) A necessary component of the LSB medium was serum. Since this was necessarily obtained in the form of whole blood from an abattoir we were faced with the tedious procedure of separation, clarification and sterilization by filtration. The final medium was also sterilized by filtration with the attendant risk of loss by contamination and of alteration of the bacterial flora of amoeba cultures when a lightly contaminated tube of medium was inadvertently used.

As Reardon and Rees(4) had shown that serum was not required in the over-lay for whole egg media and that cholesterol could substitute in part for egg yolk(5) we attempted

without success to replace the serum of LSB medium with the recommended amount of cholesterol. We therefore subjected serum to fractionation.

*Experimental.* The serum proteins were precipitated by adding 5 vol. of ethyl alcohol at 0°C. The precipitate was exhaustively washed with cold alcohol and cold acetone. The dried precipitate was redissolved in M/30 phosphate buffer in 0.4% NaCl at pH 7.2 and brought to the volume of the original serum, (Fraction "A".) The pooled filtrates were evaporated to dryness and the residue extracted with absolute ethyl alcohol. The extract was reduced to 10 ml volume and added rapidly to a volume of buffered NaCl solution equal to that of the original serum. The alcohol was removed by boiling and the original volume restored with distilled water. The resulting emulsion was Fraction "B." The alcohol-insoluble residue was extracted with acetone and an emulsion prepared as above (Fraction "C".) The 3 fractions so obtained were added to the liver-infusion base of LSB medium in proportions corresponding to 10, 5 and 1% of whole serum. The medium containing Fraction "A" was sterilized by

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2. Chang, S. L., *Am. J. Trop. Med.*, 1942, v22, 471.

3. Griffin, A. M., and McCarten, W. G., *J. Parasit.*, 1949, v35, 193.

4. Reardon, Lucy V., and Rees, C. W., *J. Parasit. Suppl.*, 1939, v25, 13.

5. Rees, C. W., Bozicevich, J., Reardon, L. V., and Daft, F. S., *Am. J. Trop. Med.*, 1944, v24, 159.

filtration, the others in the autoclave for 15 minutes at 15 lbs.

All media were inoculated with the NRS strain of *Endamoeba histolytica* using the usual technic. The cultures were incubated at 37°C, examined and transferred at 48 hour intervals with the results shown in Table I. With the extracted proteins growth failed beyond the fourth or fifth transfer on repeated trials. The alcohol-insoluble, acetone-soluble fraction was even less effective. The alcohol soluble fraction, however, was apparently capable of supporting growth indefinitely. These results suggested that steroid or phospho-lipid components of serum might be the needed factor in spite of our previous failure to get persistent growth when serum was replaced with pure cholesterol. The basic liver infusion and completed medium (LSB) were analyzed for cholesterol and cholesterol esters by the method of Hess.(6) The infusion contained 0.023 mg cholesterol per ml and no detectable esters. LSB medium had 0.071 mg cholesterol and 0.130 mg cholesterol esters per ml.

We therefore substituted 0.15 mg per ml of cholesteryl oleate or palmitate for the serum of LSB medium, sterilized the media in the autoclave and tested them with the NRS strain as above. The cholesteryl oleate medium gave results at least equal to the serum medium and was perfectly clear though slightly opalescent.

As we refined our quantitative technic it became possible to assay the individual effects of components of the medium. We therefore tested the segregated action of the cholesterol and oleic acid moieties singly and in combination. Media were prepared containing cholesteryl oleate 0.15 mg per ml, cholesterol 0.09 mg per ml, oleic acid 0.06 mg per ml and the mixture of the last two. These were inoculated in duplicate with 100,000 trophic amoebae from a 48 hour culture of a strain of *Entamoeba terrapinae*, incubated for 48 hours at 30°C, and the yields determined. Subcultures were similarly inoculated where the yield was sufficient to provide 100,000 organ-

TABLE II.  
Comparison of Cholesteryl-Oleate and Cholesterol Media.

Mean yields of duplicate cultures  $\times 10^{-4}$ .

Transfer	Media		Mean
	Cholesteryl-oleate	Cholesterol	
1	165	216	190
2	184	204	194
3	186	229	207
4	150	194	172
5	179	188	184
6	179	212	196
7	173	165	169
8	180	258	217
9	157	226	192
Mean	172	210	191

isms, otherwise the entire sediment was transferred. *E. terrapinae* has been used for most of our quantitative studies as its growth parallels that of *E. histolytica* without being as sensitive to changes in the environment.

In the media containing oleic acid alone or mixed with cholesterol there was no growth. The initial inoculum was not recoverable and the cultures were free of amoebae after the second transfer. The results obtained with the successful media are given in Table II. Direct comparison with LSB medium was not possible since it had been abandoned in favor of the cholesteryl oleate medium. However, 40 similar cultures in LSB medium selected at random from previous work gave a mean yield of  $173 \times 10^4$ . The analysis of variance gives little reason to question the greater effectiveness of the cholesterinized medium ( $F = 51.76$ ) and indicates that our previous failure to obtain growth with cholesterol alone was due to inadequate concentration. Transfers and interaction introduced some variability ( $F = 3.66$  and  $3.18$  respectively) which can be traced to instability in the cholesterinized medium. The yields in cholesteryl oleate medium are notably homogeneous in successive transfers ( $F = 0.86$ ) in contrast to those in cholesterinized medium ( $F = 9.09$ ).

The unexpected finding that oleic acid by itself and in mixture with its equivalent of cholesterol was extremely toxic led us to a study of the quantitative nature of its action. Cholesterinized liver infusion was mixed with

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TABLE III.  
Effect of Oleic Acid on Growth of *E. terrapinae*.  
Mean yields of duplicate cultures  $\times 10^{-4}$ .

Transfer	Oleic acid, mg/ml						Mean
	0.00	0.01	0.02	0.03	0.04	0.05	
1	510	694	765	610	498	293	560
2	438	377	819	430	202	361	438
3	460	387	670	240	169	174	350
4	514	307	515	452	274	235	383
5	404	371	593	370	288	178	368
Mean	464	427	672	420	286	248	420

graded amounts of oleic acid up to 0.05 mg per ml and the media inoculated with 100,000 amoebae as before. The results over successive transfers are given in Table III. The highly significant effects are associated with concentrations of oleic acid ( $F = 33.00$ ) and the comparison between the initial transfer and those following ( $F = 45.01$ ). The slight heterogeneity existing among the second through fifth transfers and their interaction with concentration ( $F = 2.64$  and  $2.28$  respectively) reflects the progressively poorer yields with oleic acid concentrations other

than optimum.

**Discussion.** Our initial failure to substitute cholesterol for serum in LSB medium appears to be due to a quantitative effect. When supplied with less than some undetermined minimum amoebic growth fails. The stimulating effect of oleic acid in optimum concentration (0.02 mg per ml), together with the smaller yields obtained with cholesteryl oleate, suggests that cholesteryl oleate as such is not available to the amoebae but must be hydrolyzed to its constituent molecules.

**Summary.** Cholesterol in adequate amounts can be substituted for serum in liver infusion media for culture of entozoic amoebae, but the yields will be statistically heterogeneous in contrast with those obtained when cholesteryl oleate is used as a supplement. Oleic acid in optimum concentrations reinforces the action of cholesterol. It is suggested that cholesteryl oleate is not used as such but must be hydrolyzed to its constituent molecules.

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## Beneficial Effects of Liver on Growth and Survival of Immature Rats Fed Lactose-Containing Diets. (17530)

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Available data indicate that the deleterious effects of rations containing high levels of lactose are dependent in considerable degree on the composition of the diet employed. Boutwell *et al.* (1) observed that rats fed a purified ration containing 48% lactose and 28% corn oil developed an unthrifty appearance and extensive diarrhea during the first 6 to 12 days of feeding. A similar ration containing 28% butter fat in place of the corn oil resulted in better growth, larger food intake and a good outward appearance. Furthermore, animals fed the butter fat ration had considerably less diarrhea than those on the corn oil diet. (2)

A similar difference between animals fed butter fat and corn oil was observed by Ershoff and Deuel (3) in rats fed diets consisting solely of 70% lactose and 30% fat. Animals fed butter fat or margarine fat in conjunction with 70% lactose lived significantly longer than those fed a similar ration containing corn oil or cottonseed oil as the source of dietary fat. On a natural food ration, however, in which lactose was provided in the form of skim milk powder, no significant difference in growth was observed between rats fed butter

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3. Ershoff, B. H., and Deuel, H. J., Jr., *Am. J. Physiol.*, 1947, v148, 45.

1. Boutwell, R. K., Geyer, R. P., Elvehjem, C. A., and Hart, E. B., *Proc. Soc. Exp. Biol. and Med.*, 1944, v55, 153.

TABLE I.  
Composition of Experimental Diets.

Dietary component	Diet A <sub>1</sub>	B <sub>1</sub>	A <sub>2</sub>	B <sub>2</sub>	A <sub>3</sub>	B <sub>3</sub>	A <sub>4</sub>	B <sub>4</sub>
Lactose*			30.2	30.2	45.3	45.3	60.5	60.5
Sucrose	70.5	60.5	40.3	30.3	25.2	15.2	10.0	
Casein†	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0
Salt mixture‡	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5
Whole liver powder§		10.0		10.0		10.0		10.0

To each kg of the above diets were added the following synthetic vitamins: Thiamine hydrochloride, 72 mg; riboflavin, 9 mg; pyridoxine hydrochloride, 15 mg; calcium pantothenate, 67.2 mg; nicotinic acid, 60 mg; 2-methyl-naphthoquinone, 5 mg; and choline chloride, 1.2 g. Each rat also received 3 times weekly the following supplement: Cottonseed oil (Wesson), 500 mg;  $\alpha$ -tocopherol acetate, 1.5 mg; and a vitamin A-D concentrate|| containing 50 U.S.P. units of vitamin A and 5 U.S.P. units of vitamin D.

\* Milk Sugar Merck, Merck and Co., Inc., Rahway, N. J.

† Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

‡ Sure's Salt Mixture No. 1.<sup>9</sup>

§ Whole Dried Liver Powder, Armour and Co., Chicago, Ill.

|| Nopco Fish Oil Concentrate, assaying 800,000 U.S.P. units of vitamin A and 80,000 U.S.P. units of vitamin D per g.

or vegetable fats.(4-6) Findings by Boutwell *et al.*(2) indicate that the superiority of butter fat over vegetable fat on a lactose-containing diet is dependent on the B vitamin content of the diet. Animals fed butter fat gained significantly more weight than those fed vegetable fats when the basal diet was low in B vitamins. When the B vitamin content of the diet was increased, however, no significant differences in growth were observed on any of the diets employed. Boutwell *et al.*(2) suggest that butter fat may be superior to vegetable fats in promoting the intestinal synthesis of B vitamins or other nutrients essential for optimal lactose utilization. In line with the above Cary and Hartman(7) observed that liver contains a water-soluble factor (distinct from any of the known B vitamins with the possible exception of vitamin B<sub>12</sub>) which significantly increased the growth of immature rats fed a purified ration containing 27% lactose. Liver has similarly been found to exert a protective effect on the growth and length of survival of immature rats fed a diet containing 73.2%  $\beta$ -lactose.(8) The

present experiment was undertaken to obtain further data on the effects of liver feeding on growth and length of survival of immature rats fed purified rations of varying lactose content.

*Procedure and results.* Two basal rations were employed in the present experiment: diet A and diet B. Diet A was a purified ration containing the B vitamin factors in synthetic form only; diet B was similar in composition but contained in addition 10% defatted and desiccated whole liver. Both rations were supplemented with 0.0, 30.2, 45.3 and 60.5% U.S.P. lactose added in place of an equal amount of sucrose. Sixty-four female rats of the Long-Evans strain were selected at 22 to 24 days of age and an average weight of 44.7 g for the present experiment. Animals were placed in metal cages with raised screen bottoms to prevent access to feces and were fed *ad lib* the various diets listed in Table I (8 animals per group). Feeding was continued for 40 days or until death, whichever occurred sooner. All surviving rats were autopsied after 40 days of feeding. Ovarian weights were determined; ovaries were fixed in 10% formol, and sections were prepared and stained with hematoxylin and eosin.

Findings indicate that growth was retarded in all rats fed lactose-containing rations

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TABLE II  
Effects of Liver Feeding on Body and Ovarian Weight and the Length of Survival of Immature Rats Fed Lactose-containing Diets.

Dietary group	Lactose content, %	No. of animals	Initial body wt, g	Gain in body wt over 40 day period,* g	% surviving†	Ovarian wt,* mg
A <sub>1</sub>	0.0	8	44.5	114.6 ± 5.5 (8)	100	39.0 ± 2.9
B <sub>1</sub>	0.0	8	44.4	129.0 ± 7.0 (8)	100	48.7 ± 5.3
A <sub>2</sub>	30.2	8	45.6	96.5 ± 3.2 (8)	100	23.5 ± 3.7
B <sub>2</sub>	30.2	8	44.3	119.2 ± 5.5 (8)	100	41.0 ± 5.2
A <sub>3</sub>	45.3	8	44.6	84.3 ± 4.1 (8)	100	16.1 ± 2.7
B <sub>3</sub>	45.3	8	44.5	107.4 ± 6.0 (8)	100	36.6 ± 3.9
A <sub>4</sub>	60.5	8	44.9	44.0 ± 7.1 (2)	25	12.2 ± 1.4
B <sub>4</sub>	60.5	8	44.7	82.6 ± 2.8 (7)	87.5	17.4 ± 1.8

The values in parentheses indicate the number of animals which survived and on which averages are based.

\* Including standard error of the mean calculated as follows:  $\frac{\sqrt{\sum d^2}}{n} / \sqrt{n}$  where "d" is the deviation from the mean and "n" is the number of observations.

† Experimental period—40 days.

(Table II). Growth retardation was most marked in animals fed diet A<sub>4</sub> and B<sub>4</sub> (60.5% lactose) and was progressively less marked in animals fed smaller levels of lactose (diet A<sub>3</sub> and B<sub>3</sub>, diet A<sub>2</sub> and B<sub>2</sub>). At all levels of lactose feeding animals fed the whole liver powder gained significantly more weight than those fed a similar diet with liver omitted. This was particularly true for animals on the 60.5 per cent lactose ration. In the absence of dietary lactose, rats fed the whole liver powder (diet B<sub>1</sub>) also gained more weight than animals on the liver free ration (diet A<sub>1</sub>). The differences in growth between the latter, however, were not statistically significant. All lactose fed rats developed diarrhea during the first 10 days of the experiment. It occurred earlier and was more extensive in rats fed the higher lactose diets than in animals fed the 30.2% lactose diet. It was particularly marked on diet A<sub>4</sub>. Subsequent to this period the diarrhea decreased in severity and gradually disappeared although it persisted in 2 rats on diet B<sub>4</sub> and 1 rat on diet B<sub>3</sub> for the duration of the experiment. In general no significant differences were noted between

diet A and diet B in the incidence of diarrhea or its extensiveness for animals fed similar levels of lactose. For the first 10 days of feeding rats fed diets B<sub>2</sub>, B<sub>3</sub> and B<sub>4</sub> did not differ in body weight from corresponding animals on diet A, although animals fed diet B<sub>1</sub> gained approximately 20% more weight than rats on diet A<sub>1</sub>. Subsequent to this period, however, animals fed diets B<sub>2</sub>, B<sub>3</sub> and B<sub>4</sub> consistently gained more weight than did corresponding rats on diet A. Two rats each on diets A<sub>3</sub> and A<sub>4</sub> developed extensive alopecia during the third week of the experiment. Alopecia was not observed in other rats on these diets nor in any of the animals fed liver-containing diets. Subsequent to the 4th week of feeding new hair replaced the areas of alopecia in rats fed diet A<sub>3</sub>. The 2 rats with alopecia on diet A<sub>4</sub> succumbed. Six of the 8 rats on diet A<sub>4</sub> died during the experimental period with an average survival time for the decedents of 21.8 days (range 11-26 days). In contrast to the above only 1 rat on diet B<sub>4</sub> succumbed (8th day). All other rats survived. At autopsy a marked distension was observed in the cecum of rats fed diets containing 45.3

or 60.5% lactose. This was observed on both diets A and B. Cecal distension was also present on diets A<sub>2</sub> and B<sub>2</sub> but was less pronounced.

A significant difference in ovarian weight was observed in lactose-fed rats on the various diets (Table II). Ovaries appeared immature both in weight and microscopic appearance in animals fed diets A<sub>2</sub>, A<sub>3</sub>, A<sub>4</sub> and B<sub>4</sub>. No mature follicles or corpora lutea were present in any of these rats with the exception of 1 animal on diet A<sub>2</sub>. Six of the 8 rats on diet B<sub>2</sub> and 5 animals on diet B<sub>3</sub>, however, had ovaries which were indistinguishable grossly and in histological appearance from normal controls (diets A<sub>1</sub> and B<sub>1</sub>). In these animals mature follicles were numerous and corpora lutea were present.

Previous findings(10,11) indicate that the failure of rats to survive on diets containing high levels of lactose was due, at least in part, to an inability to hydrolyze adequately large

amounts of this disaccharide. Present findings suggest that the administration of defatted and desiccated whole liver powder increased the rats' ability to digest lactose, although a period of approximately 10 days elapsed before this effect occurred. No data are available concerning the factor or factors in liver responsible for the increased growth, survival and ovarian development on lactose-containing diets. Recent findings by Hartman *et al.* (12) suggest, however, that vitamin B<sub>12</sub> may be responsible, at least in part, for these effects.

*Summary.* The oral administration of 10% defatted and desiccated whole liver powder increased significantly the gain in body weight of immature rats fed purified diets containing 30.2, 45.3 and 60.5% lactose. It prolonged the survival time of immature rats fed a purified diet containing 60.5 per cent lactose, and it counteracted the inhibition of ovarian development which occurred on purified rations containing 30.2 and 45.3% lactose.

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### Effect of Protein Level and Inanition on Glutamine Content of Rat Tissues.\* (17531)

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A study by Roine(1) suggested the importance of glutamine in protein synthesis in yeast and indicated the need for investigation

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1. Roine, Paavo, *Suomen Keemistilehti*, 1946, v19B, 113.

of the glutamine content of blood and tissues of higher animals. Wiss(2) observed that the blood glutamine content of rats increased when the rats were placed on an inanition routine and decreased when protein was fed. These results implied that blood glutamine concentrations are governed to some extent by the level of protein ingested. This work was undertaken to relate various dietary factors to changes in glutamine concentration in animal tissues.

2. Wiss, O., *Helv. Physiol. et Pharmacol. Acta*, 1948, v6, C35.

TABLE I.  
Composition of Rations Used for Rat Dietary Studies.

Ingredient*	1	2	3	4
Casein (crude)	0%	10%	20%	40%
Sucrose	91	81	71	51
Cottonseed oil	5	5	5	5
Salt†	4	4	4	4

\* Each kilogram of ration was supplemented with the following vitamins: Thiamin, 4 mg; riboflavin, 6; pyridoxine, 3; calcium pantothenate, 20; nicotinic acid, 20; pteroylglutamic acid, 1; inositol, 20; p-amino benzoic acid, 20; choline chloride, 1 g. In addition vitamins A and D and alpha tocopherol were administered by dropper twice a week.

† Hegstead *et al.*, *J. Biol. Chem.*, 1941, v138, 460.

**Experimental.** Experimental animals were young adult male rats of the Sprague-Dawley strain. Animals were placed on a 10-day equilibration period on the control ration (Ration 3, Table I) before use. In all studies food and water were available *ad libitum*.

After the equilibration period, animals were placed on rations of varying protein levels (Table I) for a period of 10 days. During the inanition experiment the animals were allowed access only to water. At the end of the experimental periods, the animals were killed by exsanguination under light ether anesthesia, the tissues rapidly removed, frozen and stored at  $-15^{\circ}\text{C}$  until analyzed. Plasma glutamine determinations, however, were made immediately.

Glutamine was determined by nesslerization of the ammonia liberated after incubation with beef kidney preparations, essentially according to the procedure of Archibald.<sup>(3)</sup> The preparation of the tissues for the enzymatic determination involved homogenization of the weighed sample with 15 ml of 0.04 N potassium cyanide solution, dilution of the blended mixture to 20 ml with the potassium cyanide solution, and centrifugation of the resulting mixture for 10 minutes at 1500 R.P.M. Two ml aliquots (one ml in the case of liver) of the supernatant liquid were taken for each determination.

**Results.** The effect of varying the protein level upon the glutamine content of rat tissues

TABLE II.  
Relation of Protein Level of Ration to the Glutamine Content of Rat Tissues.

Tissue	Glutamine (mg/100 g or ml)			
	Ration 1	2	3	4
Plasma	3.4	3.1	2.8	3.3
Liver	189	148	97	79
Lung	174	97	74	59
Kidney	258	135	119	87
Heart	484	273	212	143
Spleen	356	172	156	136
Muscle	214	148	178	150
Protein level ingested, %	0	10	20	40

TABLE III.  
Effect of Inanition on Glutamine Content of Rat Tissues.

Tissue	Glutamine (mg/100 g or ml)				
	0 hr	12	25	49	95
Plasma	2.9	3.8	3.7	2.8	2.4
Liver	92	85	99	148	183
Lung	73	63	89	140	171
Kidney	117	122	146	200	259
Heart	212	184	219	335	418
Spleen	156	145	204	267	332
Muscle	189	162	213	246	339

is presented in Table II. Each value represents the average of two separate experiments using a total of 12 animals for each treatment.

The relation between the glutamine content of rat tissues and length of inanition is shown in Table III. After a 10-day equilibration period on the control ration (Ration 3, Table I), the animals were subjected to starvation for the indicated periods, sacrificed, and the tissues analyzed as before.

**Discussion.** These studies indicated that there was a definite relationship between the glutamine content of rat tissues and the level of protein in the ration consumed. As the protein level increased, all tissues showed a decrease in glutamine content. Muscle tissue was different than the others studied in that this decrease was not found when protein was increased above the ten percent level. In contrast to tissues generally, the plasma glutamine level declined less sharply and then rose again; at 40 percent protein intake, values similar to those of animals consuming the protein deficient ration were found.

In the tissues of rats which have been

on an inanition regimen there was a definite initial trend towards a decreased glutamine content with deprivation of food. This trend was reversed, however, after 24 hours; and after 95 hours starvation, values similar to those found in rats subsisting on a protein-free ration were attained. In contrast to the report of Wiss(2) plasma glutamine levels did not increase, in fact, there was a suggestion of somewhat decreased values at 95 hours.

Both of these observations suggest that glutamine may have a function in the catabolism of amino acids, regardless of whether the source is dietary or from the body tissues. Whether this function is a direct one involving the utilization of glutamic acid or more indirect with glutamine acting as a reservoir for the ammonia released by the deamination

of amino acids is not known. Preliminary studies in these and other laboratories do not indicate a direct conversion of administered glutamic acid to glutamine, thus suggesting that this amide may not be too closely involved in the metabolism of the corresponding dicarboxylic amino acid.

*Summary.* The variation of glutamine content of rat tissues with protein level or period of inanition has been studied. Glutamine levels showed a marked increase as the protein level of the diet decreased. An increased glutamine level was also observed with inanition. These observations indicate the possible importance of glutamine in protein metabolism.

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### Conversion of Tryptophane to Nicotinic Acid by *Trichophyton equinum*.<sup>\*</sup> (17532)

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A strain of *Trichophyton equinum*, one of the faviform trichophytons which commonly causes ringworm in horses, was found to require nicotinic acid.(1) *T. equinum* (Ged-  
oelst) is a member of the group of large-spored, ectothrix trichophytons of animal origin which may infect man. They are characterized by their poor growth on the usual sugar-peptone media on which they produce slow-growing, glabrous, or slightly downy colonies composed largely of irregular hyphae and chlamydospores. By the use of natural carbohydrate media, Langeron and Milochévitch(2) and later Lebasque(3) showed that the growth and production of

spores by these strains could be stimulated by better nutrition, and it was later shown(4,5,1) that many of the species in this group required certain vitamins and other growth factors present in natural substances. The requirement in most instances has been for thiamine and inositol, and in one case for pyridoxine as well. This Baudet strain of *T. equinum* obtained from the Culture Bureau for Fungi, Delft, Holland is the only strain known to show a requirement for nicotinic acid.

*Experimental.* No growth of this strain could be obtained on a vitamin-free basal agar prepared as follows: 50 g dextrose (C.P.), 2.0 g  $\text{NH}_4\text{NO}_3$  (C.P.), and 0.5 g  $\text{MgSO}_4$  (C.P.) dissolved in one liter of distilled water buffered to pH 7 with Sorenson's phosphate

<sup>\*</sup> This study was made with the help of a grant-in-aid from the United States Public Health Service.

<sup>†</sup> Present address: Communicable Disease Center, Mycology Laboratory, Chamblee, Ga.

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2. Langeron, M., and Milochévitch, S., *Ann. de parasitol.*, 1930, v8, 465.

3. Lebasque, J., *Ann. de parasitol.*, 1934, v12, 418.

4. Robbins, W. J., Mackinnon, J. E., and Ma, R., *Bull. Torrey Club*, 1942, v69, 509.

5. Mackinnon, J. E., and Artagaveytia-Allende, R. C., *J. Bact.*, 1948, v56, 91.

mixture ( $\text{KH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$ ). To this is added 1.5% purified agar, prepared according to the method of Robbins.(6) Addition of nicotinic acid to such a medium allows rapid growth of the fungus. Nicotinic acid may be substituted for by equal amounts of nicotinic amide, and by comparatively large amounts of the amino acid l-tryptophane.

All vitamins and amino acids were prepared in water solutions, sterilized by filtration, and added to the melted, partially cooled basal agar. Except for nicotinic acid and its amide, none of the other vitamins tested: thiamine, pyridoxine, para-amino-benzoic acid, i-inositol, choline, pantothenate, and folic acid showed any stimulatory effect when added singly or in combinations to the basal agar. The vitamins were added in amounts of 0.1 mg to 5 ml of the basal agar except for biotin where 0.05 mg was added. Twenty-two amino acids: glycine, dl-alpha alanine, beta alanine, dl-serine, dl-threonine, dl-valine, l-leucine, dl-norleucine, dl-aspartic acid, l-glutamic acid, l-lysine, l-arginine, l-histidine, l-cystine, dl-cysteine, dl-methionine, l-proline, dl-tryptophane, l-tyrosine, dl-phenylalanine, and l-hydroxyproline were tested singly and in combination in M/1000 dilution in the basal agar (with the exception of cystine which is highly insoluble, and was tested in more dilute solution). Of these only tryptophane showed any stimulatory effect.

The minimum effective concentration of nicotinic acid or amide was determined by diluting these substances serially (by halving) in tubes containing 5 ml of  $\text{NH}_4\text{NO}_3$  basal broth (prepared similarly to the  $\text{NH}_4\text{NO}_3$  basal agar described above except that no agar is added) and inoculating with washed fragments of a culture of *Trichophyton equinum* grown in a 1.0% peptone nutrient broth. Both nicotinic acid and nicotinic amide were active to a dilution containing 0.25  $\mu\text{g}$  in 5 ml  $\text{NH}_4\text{NO}_3$  broth. No growth occurred in higher dilution or in control tubes which contained no vitamin. By a similar titration it was determined that l-tryptophane was active to a dilution containing 625  $\mu\text{g}$  in 5 ml  $\text{NH}_4\text{NO}_3$  broth. dl-tryptophane showed only

half this activity indicating that only the l form was active.

This substitution of the amino acid for nicotinic acid suggests the possibility of tryptophane as a precursor of this vitamin. In view of these findings, it was of interest to determine whether the strain of *T. equinum*, a naturally occurring nicotinicless fungus, could be shown to have the ability to convert tryptophane to nicotinic acid.

A series of 150 ml Erlenmeyer flasks each containing 50 ml  $\text{NH}_4\text{NO}_3$  broth (described above), and a similar series containing 50 ml of the basal broth prepared without  $\text{NH}_4\text{NO}_3$  were inoculated with small fragments of the fungus which had been grown for 7 generations on slants containing 1000  $\mu\text{g}$  l-tryptophane in 5 ml  $\text{NH}_4\text{NO}_3$  basal agar. Varying amounts of l-tryptophane were added to the flasks in duplicate series. One series was autoclaved immediately and served as control. The fungus grew in all of the non-autoclaved flasks containing tryptophane, but no growth occurred in control flasks of  $\text{NH}_4\text{NO}_3$  basal broth. The amount of growth was roughly proportional to the amount of tryptophane present although in flasks where the  $\text{NH}_4\text{NO}_3$  had been omitted and tryptophane was the only source of nitrogen, the amount of growth was much less.

After 6 weeks the entire growth and culture medium of each flask was hydrolyzed and biologically assayed for nicotinic acid with the *Lactobacillus casei*.† The results indicated

TABLE I.

Media*	Nicotinic acid assay†	
	Tests/ $\mu\text{g}$	Controls/ $\mu\text{g}$ ‡
50 ml basal broth +		
25 mg l-tryptophane	2.32	—
5 mg l-tryptophane	2.02	.0085
5 mg l-tryptophane +	1.18	.008
100 mg $\text{NH}_4\text{NO}_3$		

\*Basal broth—5% dextrose (C.P.) + 0.01%  $\text{MgSO}_4$  (C.P.) in  $\text{H}_2\text{O}$  solution of M/15 phosphate buffers ( $\text{KH}_2\text{PO}_4$  and  $\text{Na}_2\text{HPO}_4$ ) at pH 7.

All flasks inoculated with *T. equinum* and maintained at room temperature 6 weeks before testing for nicotinic acid.

† Controls—media autoclaved immediately following inoculation with *T. equinum*.

‡ Nicotinic acid—determined by biological assay with *L. casei*.

§ The biological assay was very kindly performed by Dr. Ray F. Dawson of the Botany Department of Columbia University.

that nicotinic acid had been produced by the fungus in flasks containing tryptophane. The similar flasks which had been autoclaved immediately after inoculation with the fungus showed only negligible traces of nicotinic acid. (Table I). It is interesting to note that although twice to 3 times as much growth occurred in the tryptophane flasks which also contained  $\text{NH}_4\text{NO}_3$ , less nicotinic acid could be detected in these flasks. It seems possible that in the presence of readily available nitrogen, much of the nicotinic acid produced from the tryptophane was utilized as readily as it was formed.

**Discussion.** Krehl *et al.* (7) in their studies of the pellagragenic effect of corn were the first to show a possible interchangeable role of nicotinic acid and tryptophane. Later experiments by Rosen *et al.* (8) and Sarett and Goldsmith (9) indicated that tryptophane may be an important precursor of nicotinic acid in rats as well as in humans, and may explain the anti-pellagragenic effect of certain foods such as milk which are low in nicotinic acid but rich in protein. Bonner and Beadle (10) have described 3 x-ray mutants of *Neurospora* which require nicotinic acid for growth, and in a later communication, Beadle, Mitchell, and Nye (11) have shown that one of the nicotinicless mutant strains, No. 65001, was able to grow when supplied with either tryptophane or nicotinic acid. By means of another nicotinicless mutant strain which could not utilize tryptophane, they further showed that strain No. 65001 actually had the ability to convert tryptophane to nicotinic acid. Nicotinic acid could also be produced by this strain from dl-kynurenine, a substance which has been known as a product of tryptophane metabolism in animals and bacteria, and is a possible intermediate in the conversion of

tryptophane to nicotinic acid. On the basis of the growth responses of this *Neurospora* mutant strain to tryptophane, nicotinic acid, and kynurenine, it was postulated that nicotinic acid is normally formed from tryptophane with kynurenine as an intermediate.

According to Schopfer, (12) no naturally occurring fungi have been shown to require nicotinic acid. Whether the strain studied is a normally occurring type or an unusual mutant form is not known, and it is doubtful that the requirement for nicotinic acid by this strain of *T. equinum* is a characteristic which would be of value in species determination. The only other strain of *T. equinum* available for testing was No. 9870 from the American Type Culture Collection. This strain did not require nicotinic acid, but did require thiamine and i-inositol. In this respect it resembles *T. faviforme*, a common agent of ringworm in both cattle and horses. Since the separation of *T. equinum* from *T. faviforme* depends on the insecure grounds of its more frequent isolation from horses than from cattle and its ability to produce more aerial mycelium on the surface of colonies on Sabouraud's dextrose agar, it seems highly probable that *T. equinum* is synonymous with *T. faviforme*. Further strains must be obtained and studied in order to determine whether the requirement for nicotinic acid is present in any other strain of this group.

The transformation of tryptophane to nicotinic acid by the strain *T. equinum* (Geddoelst) gives further evidence for the hypothesis that, in both plants and animals, tryptophane is a precursor in the formation of nicotinic acid, essential to the growth of these forms.

**Summary.** A strain of *Trichophyton equinum* has been shown to have a requirement for nicotinic acid. Nicotinic acid may be substituted for by equal amounts of nicotinic amide and comparatively large amounts of l-tryptophane. The fungus has been shown to have the ability to convert tryptophane to nicotinic acid, thus giving further evidence in support of the hypothesis that tryptophane may be a precursor of nicotinic acid.

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# Prodigiosin. I. Antibiotic Action on *Coccidioides immitis* in Vitro.\* (17533)

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Prodigiosin is a thermostable, water insoluble, red pigment produced by *Serratia marcescens* under certain conditions. The dye has stimulated interest because of its ability as a contaminant to discolor water and food. This characteristic is better known than its inhibitory action against microbial agents.

The antibiotic properties of prodigiosin recorded in the literature indicate a possible wide spectral range of activity against bacteria and protozoa. No evidence concerning its fungistatic action could be found. Hettche(1) first reported the bacteriostatic activity of prodigiosin for staphylococci and *Bacillus anthracis* in vitro. The growth of *B. anthracis* was inhibited in a dilution of 1:200,000 after 3 hours' exposure and a dilution of 1:25,000 appeared effective against staphylococci after one hour's exposure. He was unable to demonstrate any activity against the gram-negative organisms, *Escherichia coli* and *Salmonella paratyphi*. Fischl(2) demonstrated that prodigiosin would protect mice against nagana. A single subcutaneous injection of 10 mg of prodigiosin in sweet almond oil caused the disappearance of *Trypanosoma brucei* from the peripheral blood.

Eisler and Jacobsohn(3) demonstrated that sterile bouillon extracts of *S. marcescens* inhibited the growth of *Corynebacterium diphtheriae* and of *Neisseria gonorrhoeae*. Their results, however, were not directly related to

dye content. Singh(4) reported toxic effects of the pigment of *S. marcescens* on soil amoeba. The concentration of the pigment which prevented amoebae from consuming an edible substrate of bacteria was not reported.

The naturally occurring dye was first isolated by Kraft,(5) who called it "prodigiosine." Wrede, Hettche, and Rothhaas(6) presented a series of articles on the chemical properties of prodigiosin and identified the structure as a tripyrrole methane.

**Materials and methods.** Abundant pigment production was obtained from growth of *S. marcescens* on thick mannite agar plates (15.0 cm) adjusted to pH 5.0.† Surface inoculation with a single strain of *S. marcescens* (Stanford Z-4 strain) was followed by incubation at 22°C for from 5 to 7 days. The surface growth was harvested by scraping and contained large amounts of both intracellular and extracellular red pigment which was extracted by a modification of Wrede's method (Lack and Botts).(7) Yields of from 1.0 to

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†Mannite agar formula:

Mannite	20 g
Neopeptone	10 g
Meat extract	3 g
MgSO <sub>4</sub>	1.25 g
Brewer's yeast	1 g
Agar	20 g
Q.S. and H <sub>2</sub> O	1000 cc
Adjusted to PH 5.	

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\* Sponsored by the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are a result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

1. Hettche, H. O., *Arch. Hyg.*, 1932, v107, 348.

2. Fischl, V., *Z. f. Immunitats.*, 1935, v85, 77.

3. Eisler, M., and Jacobsohn, I., *Z. f. Hyg. Infekt.*, 1936, v117, 76.

TABLE I.  
Fungistatic and Fungicidal Activity of Prodigiosin Base and Certain Salts on *Coccidioides immitis* *in vitro*.

Derivative	Fungistatic conc., $\times 1000$	Fungicidal conc., $\times 1000$
Base, purified	1:500	1:100
Hydrochloride	1:500	1:100
Perchlorate	1:500	1:100
Glutamic acid (water soluble)	1:250	1:83
Tartrate	1:100	1:50
Sulfonate	1:10	0
Sulfonamide	1:1	0

2.0 mg of prodigiosin were obtained from approximately 10 sq cm of wet surface growth. Repeated purifications yielded a prodigiosin from which several new compounds were made.<sup>‡</sup> Each compound was tested *in vitro* for its fungistatic and fungicidal action against *Coccidioides immitis* (Strain S.F.S. 46). Inasmuch as prodigiosin base is insoluble in water, tests were made to determine the most satisfactory solvent. Propylene glycol and ethanol were found to be suitable vehicles for *in vitro* and *in vivo* tests. Propylene glycol is sufficiently miscible in water to permit uniform dilutions of the derivatives to be tested in liquid synthetic culture medium.

Serial dilutions of prodigiosin in propylene glycol or ethanol from 1:10,000 to 1:10,000,000 were made in a synthetic medium.<sup>§</sup> Controls with equivalent amounts of propylene glycol or ethanol in this medium were run in parallel. Each tube was inoculated with a 0.1 ml of a known concentration of chlamydospores which was diluted to give a range of from 2.0 to 3.0  $\times 10^4$  spores. After incubation of 24 and 48 hours at 34°C 0.1 ml was transferred from each tube to 9.9 ml of the

liquid synthetic medium as a test for viability. The subcultures were incubated at 34°C and inspected for growth after from 10 to 14 days on a basis of no growth (0) to abundant growth (++), indicating first signs of spore germination to rich mycelial development.

**Results.** The prodigiosin first prepared was fungistatic in a dilution of 1:250,000. Subsequent purifications increased the activity to 1:500,000 (Table I). Fungicidal activity as determined by the subcultures showed no growth at 1:100,000 dilutions. Propylene glycol controls revealed no inhibition of *C. immitis* except in the first tube of the series containing 10% propylene glycol. The hydrochloride and perchlorate salts of prodigiosin base inhibited growth in a dilution of 1:500,000. The tartrate maintained moderate effectiveness; the sulfonate and sulfonamide derivatives showed an almost complete loss of activity. A water soluble preparation was made by fusion of glutamic acid with prodigiosin base. This glutamic acid compound demonstrated effective fungistatic activity at a 1:250,000 dilution.

A comparison was made of the fungistatic and fungicidal effectiveness of prodigiosin purified bases when dissolved in propylene glycol or ethanol. Inhibition of growth of *C. immitis* was obtained in a dilution of 1:500,000 in both series. Although definite inhibition occurred at a dilution of 1:1,000,000, after several weeks' incubation germination appeared first in the propylene glycol and then the alcohol series. The propylene glycol and alcohol controls showed abundant growth in all except the initial tubes containing equivalent amounts of diluent. The tubes were subcultured at 48 hours to test the fungicidal activity. Slightly better results were obtained

<sup>‡</sup> The preliminary investigation of the chemical constitution and general properties of prodigiosin were performed with the assistance of Dr. Frederick Proescher and Mr. V. Sycheff, formerly of the Santa Clara County Hospital laboratory. Subsequent extension of similar chemical research has been with the aid of Dr. Elbert Botts in the laboratory of the Birmingham Veterans Administration Hospital, Van Nuys, Calif.

<sup>§</sup> Synthetic media:

NH <sub>4</sub> Cl	g
Na Acetate	10
KH <sub>2</sub> PO <sub>4</sub>	10
K <sub>2</sub> HPO <sub>4</sub>	2
H <sub>2</sub> O q.s. ad 1000 cc pH 6.12.	6



in the propylene glycol series. Complete loss of viability of all chlamydozoospores after 48 hours' exposure was established at a prodigiosin dilution of 1:100,000.

**Summary.** Prodigiosin was fungistatic for *Coccidioides immitis* in dilutions through 1:500,000. It was fungicidal after 48 hours'

exposure in dilutions through 1:100,000. Prodigiosin hydrochloride, perchlorate and a water soluble glutamic acid derivative demonstrated similar *in vitro* effectiveness.

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## Pregnancy in Alloxan Diabetic Rats. (17534)

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Because of the well recognized frequency of complications in the course of pregnancy in clinical diabetes, this study was undertaken to determine the course of pregnancy in rats suffering from uncontrolled chronic diabetes. Since August 1946, when these studies were begun, 4 reports(1-4) have appeared dealing with this subject each with a slightly different approach to the problem and each with somewhat varying results.

**Methods.** Twenty-seven 70-90 day old Wistar strain female rats were fasted overnight and given a single subcutaneous dose of alloxan varying from 140-175 mg per kg. Where a single dose failed to produce a persistent diabetic state, repeated injections of increasing doses were employed up to 205 mg per kg. In some rats it was necessary to administer as many as nine doses of alloxan in order to achieve permanent diabetes. In 2 rats permanent diabetes did not appear despite repeated injections of alloxan. During the entire period covered by these studies the animals were kept in individual metabolism cages and the following data were obtained daily, 7 days a week, for periods up to 448 days: (a) total fluid intake, (b) total food intake, (c) total urine volume, (d) total quan-

titative glycosuria, (e) qualitative acetonuria, (f) vaginal smears for determination of oestrus. The animals were given free access to a constant mixture of crushed Purina Lab Chow (Protein 25%, Carbohydrate 50%, Fat 6.3%) reinforced with the following: reduced iron 0.2 g; sodium chloride (iodine-free) 10 g; sodium chloride (iodized) 2.7 g; riboflavin 5 mg, vitamin B<sub>6</sub> 5 mg, vitamin D 3000 U.S.P. units, vitamin E 100 mg, choline 15 mg, per kilo of chow in order to bring the ration up to an adequate level for breeding as recommended by the Ralston Purina Company.

Because of the frequent occurrence of a transient diabetic state after alloxan injection, matings were performed when possible only after the establishment of permanent diabetes by placing one young adult male of the same strain into the metabolism cage with each female on the day of oestrus. Animals were considered definitely diabetic only when they fulfilled both of the following criteria: (1) persistent glycosuria greater than 0.5 g per 24 hours as measured by Benedict's Picrate method, and (2) repeated non-fasting venous blood sugars in excess of 200 mg% obtained from tail blood or cardiac puncture and measured by the technique of Lauber and Mattice.(5) Fasting blood sugars were not employed in this study, because it was thought that repeated overnight fasts might introduce an irregular influence on the nutritional or

1. Davis, M. E., Fugo, N. W., and Lawrence, K. G., *Proc. Soc. Exp. Biol. and Med.*, 1947, v66, 638.

2. Miller, H. C., *Endocrinology*, 1947, v40, 251.

3. Hultquist, G., *Acta Path. et Microbiol. Scand.*, 1948, v25, 131.

4. Sinden, J. A., and Longwell, B. B., *Proc. Soc. Exp. Biol. and Med.*, 1949, v70, 607.

5. Lauber, F. V., and Mattice, M. R., *J. Lab. and Clin. Med.*, 1944, v29, 113.

diabetic state of the animals. In those animals considered permanently diabetic, with two exceptions (c.f. Rats no. 18 and no. 19 below) the criteria for diabetes remained satisfied until the animals were either sacrificed, died, or were removed from the experiment after periods varying from 32 to 425 days.

**Results.** Of the 25 rats in which permanent diabetes was produced, 12 pregnancies were achieved in 7 rats, 2 of which had 3 pregnancies, 1 had 2 pregnancies and the remainder 1 each. Of the 12 non-diabetic control rats of the same age and strain as the experimental animals, 11 pregnancies were produced in 8 rats over the same period of time. Despite the absence of any effort at treatment of the diabetes, clinical acidosis was not observed in any of the 25 permanently diabetic rats. In no case was insulin administered. Nevertheless, most of the animals remained in apparent good health, with maximum 24 hour water intakes of 200-400 cc and maximum urine outputs of 70-140 cc. Occasional isolated instances of acetonuria were observed. When olive oil was added to the diet, however, acetonuria was frequent and persistent. No blood carbon dioxide studies were performed, but clinical evidence of acidosis failed to appear. Blood sugars in the control group taken under the same non-fasting conditions as in the diabetic group averaged 95 mg% with variation from 79 mg% to 119 mg%. Diabetic animals revealed blood sugars varying from 224 mg% to 650 mg%, with a single exception of one value of 133 mg%. The average of all diabetic blood sugars was 450 mg%.

As previously reported,<sup>(1,4)</sup> there was disappearance of oestrus vaginal smears for long periods of time and irregular oestrus cycles appeared in females with well established diabetes. In rats made diabetic by subtotal pancreatectomy Richter *et al.*<sup>(6)</sup> reported the return of normal oestrus activity, when these animals ingested a high fat diet. The main constituent of this high fat diet was olive oil. Richter's observations are in large measure

confirmed in our studies. Animals partook of olive oil when it was offered and there was a definite trend toward normal oestrus cycles as a result thereof. In most cases, however, irregularities persisted and in no case did pregnancy occur after the use of olive oil.

Observation of these 12 diabetic and 10 normal pregnancies revealed the following data: (1) The estimated period of gestation varied between 20 and 29 days in the diabetic group and between 20 and 28 days in the control group, with the majority of both groups varying between 20 and 23 days. (2) No significant alteration in the severity of the diabetes was noted during pregnancy or in the puerperium in any of the animals, with the 2 exceptions, c.f. Rats no. 18 and no. 19 below. (3) Though urines were not tested routinely for albumin and no blood pressure studies were performed, there was no clinical evidence of toxemia and the pregnancies in the diabetic animals appeared quite as normal as those of the controls. (4) Figures for the number of young per litter as determined by observation were found to be unreliable both in the diabetic and control series, because of the tendency in both groups to some degree of cannibalism. In spite of this, however, no significant difference was noted in the number of offspring per litter in the two groups. (5) No external congenital anomalies were observed at birth in any litters in either the experimental or control series and, so far as clinical observation could determine, the fetuses were grossly of equal size in the two groups. Four offspring of 2 diabetic animals were observed to maturity and these animals proved to be completely normal in all respects. Repeated blood sugar levels were determined in these offspring of diabetic mothers after weaning at 28 to 35 days and no deviation of the blood sugar from normal was observed. (6) Because of the size of the newborn rat it was impossible to measure the blood sugar level and since a clinical diagnosis of hypoglycemia under these conditions was considered valueless, no data were obtained on this phase of the subject. (7) Compared with their pre-pregnant course and the diabetes of other non-pregnant rats, no alteration in the course of the disease was noted in 5 rats subsequent to

6. Richter, C. P., Schmidt, E. C. H., Jr., and Malone, P. D., *Bull. Johns Hopkins Hosp.*, 1945, v76, 192.

TABLE I.  
Data on Pregnant Diabetic Rats.

Rat No.	Duration of diabetes (days)	Alloxan		Glycosuria (non-pregnant period) avg*	Blood sugars (non-pregnant period) range, mg%	Duration of diabetes prior to pregnancy days	Glycosuria (pregnancy) avg*	Blood sugars (pregnancy) (mg%)	Avg
		Doses	mg/kg						
8	351	6	140	4.15 (0.19-12.6) †	330-598	(1) 27 (2) 100 (3) 145	(1) 2.99 (12-6.7) † (2) 3.40 (.57-6.3) (3) 4.20 (.44-11.8)	(1) 290-432 (2) 400-580 (3) 432 (1)	392 480
16	425	8 1	140 165	0.95 (.00-12.6)	270-522	(1) 50 (2) 124 (3) 207	(1) 0.75 (0-4.0) (2) 2.87 (0-8.1) (3) 2.64 (0-8.0)	(1) 224-400 (2) 380-488 (3) No blood sugar	368 484
17	405	5 2 1	140 180 200	2.85 (.00-5.3)	236-650	(1) 23 (2) 134	(1) 0.58 (0-3.1) (2) 4.53 (0-12.1)	(1) 340-400 (2) 402-480	375 471
18	68	3	140	1.28 (.00-2.5)	300-444	53	1.55 (.28†-3.7)	368 and 26 (2)	
19	32	5 1 1	140 180 205	1.1 (.48-1.7)	250 (1)	4	1.02 (0-4.0)	444 and 810 (2)	
23	130	1	170	2.56 (.82-5.2)	345-444	4	1.75 (0-6.1)	328 and 133   (2)	
27	274	2	175	4.23 (1.2-11.6)	246-456	16	1.52 (.27-3)	380 single	

\* g/24 hr. † Range. ‡ 0 on day of death. || Day before delivering.

single or repeated pregnancies. (8) In Rats No. 18 and 19 of this series a phenomenon was observed which we are at a complete loss to explain. Both of these animals died in hypoglycemia. Neither of them was given insulin at any time. Rat No. 18 was first noted to be somewhat apathetic on the 15th day of her only pregnancy, 68 days after the onset of her diabetes, which prior thereto had not differed from that observed in our other animals. Blood sugar 13 days before death was 368 mg%. Glycosuria in excess of 0.5 g in 24 hours had persisted until the day before death. Shortly after the apathy appeared cyanosis developed. There was bleeding from the nose and vagina. The coat became ruffled and the temperature fell to 99° (R). Five hours after the onset of this train of symptoms the animal was moribund. A cardiac puncture revealed a blood sugar of 26 mg%. Death ensued within 15 minutes. Autopsy was performed immediately. There were no gross abnormalities. Microscopically, the kidneys showed colloid droplet degeneration in both the proximal and distal convoluted tubules. The sections of the eyes showed the presence of incipient cataracts. Well-formed islets of Langerhans were present in sections of the pancreas. There was no necrosis or excessive fat deposition in the adrenals or liver. Sections of the other organs including the pituitary were not remarkable.

Rat No. 19 died with identical symptoms 6 days after delivery of a litter of 9 living young, 32 days after the onset of her disease. Her diabetes had likewise run the usual course. Blood sugar 16 days prior to death was 810 mg%. Following delivery the animal ate no chow and drank little water but devoured her entire litter. She became moribund 8½ hours after the onset of symptoms. Cardiac blood sugar at this time was 22 mg%. Death occurred about 15 minutes later and autopsy was performed immediately. No gross abnormalities could be made out. Microscopically, the only positive finding was the presence of incipient cataracts in sections of the eyes.

**Discussion.** The results of this study of 12 diabetic and 10 control pregnancies confirm previous reports(1,4) that alloxan diabetes, like pancreatectomy diabetes(6) interferes

with normal oestrus. Administration of olive oil to these animals tends to restore a more normal oestrus pattern as demonstrated by Richter(6) for subtotally pancreatectomized animals, but does not influence fertility.

Our results are contrary to those of Davis *et al.*(1) who reported the resorption of fetuses in 4 uncontrolled alloxan diabetic rats and stated that control of the hyperglycemia by insulin, as demonstrated in 7 treated rats, was essential to successful completion of pregnancy with live litters.

Miller(2) reported 10 normal pregnancies in 8 diabetic rats where the average glycosuria per day varied from 0.27 g to 0.5 g, in 3 untreated animals, and from 0.3 g to 1.6 g per day in 7 rats treated with protamine zinc insulin in order to prevent severe glycosuria. No blood sugars were reported. These untreated animals demonstrated mild diabetes as judged by our requirement of a glycosuria in excess of 0.5 g in 24 hours.

Davis *et al.*(1) reported that in untreated alloxan diabetic rats "pregnancy progressed normally until about the 12th day following which the fetus died and was slowly absorbed". Our data, however, do not support this finding and confirm those of Sinden and Longwell.(4) Eleven of the 12 pregnancies studied progressed normally to term with the delivery of normal litters. The need for insulin as stressed by Davis *et al.*(1) did not exist in this series. In fact, the only rat that died during pregnancy manifested a terminal hypoglycemia.

**Summary and conclusions.** (1) Alloxan diabetes, like pancreatectomy diabetes, interferes with the normal oestrous cycle of rats, and impairs fertility.

(2) Administration of olive oil aids in reversion of the oestrous cycle toward normal, but does not improve fertility.

(3) The course of 11 of the 12 pregnancies reported in this study was uninfluenced by the presence of pre-existing permanent alloxan diabetes.

(4) The course of the diabetes in 5 of the 7 rats observed was not influenced by single or repeated pregnancies.

(5) Two rats died in hypoglycemia, one on the 15th day of pregnancy and one 6 days

post partum. The mechanism of this hypoglycemia is unexplained.

(6) Administration of insulin was not re-

quired in order to obtain live, full term litters in 10 of the 11 pregnancies observed.

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## Purification of Certain Viruses by Use of Protamine Sulfate. (17535)

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During attempts to improve the immunogenic potency and to increase the purity of Japanese encephalitis vaccine, it was noted that the addition of protamine sulfate to suspensions of infected chick embryo tissue produced a heavy precipitate, and, of particular interest, left almost all of the virus in the clear supernatant fluid. Subsequently, a number of viruses were studied with respect to their behavior in the presence of protamine. It was found that they fell into 2 groups, *i.e.*, those which were precipitated along with the tissue materials as has been observed by others (1,2) and those, like Japanese encephalitis virus, which remained in the supernatant fluid. For this latter group, protamine precipitation provides a rapid, simple and effective means for partial purification.

**Methods.** A considerable number of orienting experiments indicated that protamine precipitation could be carried out in the following manner: A chilled infected 10% suspension of mouse brain clarified by low-speed centrifugation or a 20% suspension of uncentrifuged chick embryo tissue, pH 7.0 to 7.2, is added to protamine sulfate (salmine) in the proportion of 1.0 ml of tissue suspension for each 5.0 mg of protamine. Either the readily soluble dry protamine powder or a solution of the material is used. A fine precipitate forms immediately on the addition of the tissue suspension to the protamine. The mixture is stored with occasional agitation in the refrigerator for 30 minutes after which the pre-

cipitate is separated by low-speed centrifugation at 3000 RPM for 15 minutes in an angle centrifuge. The resultant clear, pink supernatant fluid is decanted and saved; in the present report it is designated as "protamine supernate." Protamine supernates obtained by the method just described develop a granular precipitate during the first 24 hours of storage in the refrigerator. Furthermore, if formaldehyde is added in order to inactivate the virus, a heavy flocculation slowly develops. This flocculation apparently is due to the residual protamine in the preparations. It can be avoided if the excess protamine is removed by precipitating it with heparin. The optimum amount of heparin for this purpose is 3.8 mg per ml of supernate. Less than this amount fails to remove sufficient protamine whereas more produces a finely dispersed precipitate which sediments with difficulty. Thirteen viral agents were subjected to treatment with protamine. Their distribution in the various fractions thus obtained was determined by assaying the infectivity of the materials. In all but 2 instances this was done by intracerebral titration in mice in the usual manner. The exceptions were mumps virus, which was assayed in embryonated eggs by testing for the presence of viral hemagglutinins in the chorioallantoic fluids, and one strain of vaccinia virus, which was titrated on the chorioallantoic membrane.

**Results. Action of protamine on infected suspensions.** The viral agents which remain in the clarified supernatant fluid after treatment with protamine are listed in Table 1, as are those which are precipitated along with tissue materials by this treatment. Others

1. Chambers, L. A., and Henle, W., *Proc. Soc. Exp. Biol. and Med.*, 1941, v48, 481.

2. Bawden, F. C., and Pirie, N. W., *Proc. Roy. Soc., Series B*, 1937, v123, 274.

TABLE I.  
Infective Titer of Crude and Protamine-treated Suspensions of Virus Infected Tissues.

Virus	Source	Infective titer		
		Crude suspension	Protamine-treated Supernate	Sediment
	Agents not precipitated			
Colorado tick fever	Mouse brain	6.2	5.2	3.8
Equine encephalomyelitis (western type)	" "	8.4	8.2	N.D.
Encephalomyocarditis	" "	8.5	8.5	N.D.
Japanese encephalitis	" "	8.4	8.4	<6.0
Polioomyelitis (Lansing)	Chick embryo	7.3	7.2	<6.0
	Mouse brain	3.4	3.2	N.D.
	Cotton rat brain	3.0	3.0	N.D.
Russian Spring-Summer encephalitis	Mouse brain	8.7	8.5	N.D.
St. Louis encephalitis	" "	6.7	6.8	N.D.
West Nile virus	" "	7.6	7.6	N.D.
	Agents precipitated			
Herpes	Mouse brain	4.3	<1.5	3.8
Lymphocytic choriomeningitis	" "	5.2	<2.5	5.2
Murine encephalomyelitis (GD VII)	" "	7.4	<4.0	6.8
Rabies	" "	6.6	3.5	5.4
Vaccinia	" "	5.2	<3.0	N.D.
	Chick embryo	8.2	4.5	7.0

have shown that influenza(1) and tobacco mosaic(2) viruses are precipitated by protamine. Most of the observations on factors influencing the protamine reaction were made on suspensions of chick and mouse tissues infected with Japanese encephalitis virus. The amount of protein nitrogen, *i.e.*, acetic acid precipitable N, in the mouse brain protamine supernatant was about one-third that in the crude tissue suspension. When the protamine precipitation was performed at various temperatures ranging from 2°C to 30°C, there was little variation in the virus titer and total nitrogen in the protamine supernates. However, when the temperature was over 30°C, the supernates were never quite clear. We have chosen to perform the procedure in the cold to maintain maximum infectivity of the viral materials. Other observations indicated that the technic was equally effective when carried out over the pH range from 6.0 to 8.0.

*Purity of protamine clarified materials.* On the basis of infectivity and total nitrogen values the purity of preparations of Japanese encephalitis virus obtained by ultracentrifugation of protamine supernatants was appreciably greater than that of viral suspensions ob-

tained by the usual differential centrifugation procedures. In 2 experiments aliquots of a suspension of mouse brain were treated by the 2 methods. Protamine supernate prepared from 1 aliquot was centrifuged at 100,000 G for 1 hour after which the sediment was resuspended and saved. The other aliquot was first clarified by centrifugation at 10,000 G for 1 hour and then subjected to the same treatment as the protamine supernate. The total nitrogen content of the ultrasediments, expressed as gamma/ml of suspension subjected to ultracentrifugation, was 7.0 and 3.0, respectively, for the 2 protamine-treated materials, and 73 and 40, respectively, for the materials concentrated by centrifugation alone. It should be emphasized that in one experiment the infective titers of the original suspension and of the resuspended sediments obtained by both procedures were of the same order of magnitude, *i.e.*,  $10^{-7.0}$ - $10^{-8.0}$ .

Electron micrographs were made of preparations of Japanese encephalitis virus obtained from infected mouse brains by combined treatment with protamine and heparin followed by ultracentrifugation. These showed little other than numerous spherical particles with a diameter of approximately 20  $\mu$ ; the size of

TABLE II.  
Comparative Potencies of Crude and Purified Japanese Encephalitis Vaccines (10% Mouse Brain).

Vaccine No.	Purification	Minimal immunogenic dose, ml
185A	Crude	.001
185A	Protamine*	.008
172	Protamine*	.007
176	Crude	.006
176	Protamine-heparin	.007
173	" "	.005
180B	" "	.002
181	Protamine-heparin + Ultracentrifugation	.004

\* Vaccines inactivated by ultraviolet light. All other vaccines inactivated with formaldehyde.

Japanese encephalitis virus is about 15-22  $\mu$ . Similar particles were also found in sediments from suspensions of normal mouse brain prepared in the same manner. Hence, it was not possible to differentiate between the Japanese encephalitis virus and particles of the sedimentable component which were present in normal mouse brain preparations.

*Antigenicity of protamine-treated Japanese encephalitis vaccines.* Japanese encephalitis vaccines were prepared from infected mouse brain and partially purified by treatment with (a) protamine, (b) protamine and heparin, or (c) by ultracentrifugation of protamine-heparin treated materials. In most of these the virus was killed by 0.2% formaldehyde. Two lots were inactivated by irradiation with ultraviolet light. The data summarized in Table II indicate that each of these preparations when assayed in mice would meet the potency requirements established for Japanese encephalitis vaccine.(3)

One of the lots of protamine-heparin treated vaccine (180B, Table II) was tested for its immunogenic activity in human beings. Seven volunteers were given 0.1 ml of the purified material intradermally while 8 received 1.0 ml subcutaneously. All of these persons had previously received one or more courses of crude chick embryo type Japanese encephalitis vaccine. The level of Japanese encephalitis neu-

tralizing antibody before and after injection was determined by the intracerebral mouse test. Five of the volunteers had detectable amounts of specific antibody before receiving the present vaccine (neutralization indices between 100 and 650). All 5 of these showed a marked rise in titer following their booster dose with indices which now ranged between 2500 and 40,000. In addition, 6 of the 10 volunteers without detectable antibody prior to vaccination subsequently developed significant amounts of antibody with indices ranging from 100 to 15,000. The antibody response was similar in the groups who received vaccine intradermally and subcutaneously, and did not differ appreciably from that previously noted in persons receiving booster doses of the standard Japanese encephalitis vaccine by the same routes.(3)

It may be mentioned that vaccine 180B had a titer of 1:16 when used as antigen in a complement-fixation test performed according to the technic previously employed in this laboratory.(3) This is within the limits generally obtained for antigens prepared from mouse brain infected with this virus. Protamine-treated antigens of a different type have been used by Roberts(4) in a serological flocculation test for poliomyelitis.

*Summary.* Suspensions of a number of viral agents can be partially purified by treatment with protamine sulfate which precipitates much of the extraneous material leaving essentially all of the virus in the clarified fluid. Preparations obtained by this simple method have potential value as antigens, vaccines and starting material for studies on the physical and biochemical nature of selected viral agents.

3. Warren, J., Smadel, J. E., and Rasmussen, A. F., *J. Immunol.*, 1948, v58, 211.

4. Roberts, E. C., *Pub. Health Rep.*, 1949, v64, 212.

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# Cytotoxic Action of Antisera to Cell Components of Normal and Leukemic Mouse Spleens.\* (17536)

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Serologic studies, employing cytoplasmic fractions segregated from the spleens of normal and leukemic mice (1) demonstrated quantitative, but not qualitative, differences. Since nitrogen values had been made the basis for dilution of all fractions for immunization purposes, as well as for use in serologic tests, we postulated that some other antigenic component—not measurable entirely by nitrogen evaluation—could account for the quantitative differences, as expressed by the consistently higher serum titers for the leukemic fractions. The experiment was repeated with a second lot of cytoplasmic fractions, prepared in Dr. Mary L. Petermann's laboratory, and described as follows:

"The mitochondria were separated and washed in 0.88 M sucrose as in earlier experiments; (2) except that cycles of low and high speed centrifugation were employed, to insure the removal of larger as well as smaller particles. The two fractions of submicroscopic particles,  $P_1$  and  $P_2$ , were isolated from the mitochondria supernatant by centrifuging for two hours at 180,000  $\times$  gravity. The  $P_1$  fraction came from the pellets, while the  $P_2$  came from the layer just overlying the pellets. Each fraction was washed once with 0.44 M sucrose. A detailed description of these particles will be published elsewhere." (3)

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1. Dulaney, Anna Dean, Goldsmith, Yvette, Arnesen, Kristen, and Buxton, Louise, *Cancer Research*, 1949, **9**, 217.

2. Alfin-Slater, R. B., Larack, A. M., and Petermann, M. L., *Cancer Research*, 1949, **9**, 215.

3. Petermann, M. L., Larack, A. M., and Handler, R. S., in preparation.

These cell preparations were used by us for immunization of rabbits, and as antigens in complement fixation tests. In all details these procedures followed those of the earlier study. Likewise, the results reflected those of the first experiment. Further study demonstrated that these spleen antisera—both normal and leukemic—are injurious to leukemic cells upon *in vitro* exposure. Moreover, a marked difference, as measured by survival rates of mice receiving serum-treated cells, appears between the cytotoxic effects produced by the normal and leukemic antisera in suitable dilutions, differences far greater than those revealed by complement fixation procedures.

While cytotoxic properties of antisera to various tumors have been reported by Lumsden, (4) Kidd, (5) Andervort, (6) Green, (7,8) Burmeister, (9) such action of antisera on the leukemic cell has not been described.

In this laboratory however Stock§ observed that exposure of leukemic cells to anti-leukemic cell (whole cell) or anti-normal mouse tissue sera, prior to injection of a small series of mice, resulted in a high percentage of survivals. Undiluted sera were used and the survival rate in the two groups of animals was very similar.

*Materials and methods. Antisera.* The following antisera to spleen components were tested for cytotoxic action:

- 1) Mitochondria, normal and leukemic
- 2)  $P_1$ , normal and leukemic

4. Lumsden, Thomas, *Am. J. Cancer*, 1937, **v31**, 430.

5. Kidd, John G., *Science*, 1944, **v99**, 348.

6. Andervort, Howard B., and Bryan, W. Roy, *J. Nat. Cancer Inst.*, 1944-45, **v5**, 143.

7. Green, Robert G., *Proc. Soc. Exp. Biol. and Med.*, 1946, **v61**, 113.

8. Green, Robert G., *Science*, 1947, **v107**, 93.

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§ Personal communication, Dr. C. Chester Stock.



- 3)  $P_2$ , leukemic||
- 4) Nuclei, normal and leukemic(10)
- 5) Whole cell, normal and leukemic
- 6) Heterologous tissue (liver, lung, kidney)

As previously stated, all preparations were standardized on the basis of nitrogen content. All rabbits received 6 doses of antigen adjusted to contain the same amount of nitrogen.

*The source of the leukemic cells used in Tests.* The strain of lymphatic leukemic (No. 9421) was carried routinely by intraperitoneal injection of inbred mice (brother-sister matings) with leukemic cells. The donor spleen was minced finely in 19 parts of physiological saline solution and the cells filtered through cotton by aspiration into a tuberculin syringe carrying a 26 gauge needle. The number of cells per 0.1 ml was determined by counting in a haemocytometer. Following intraperitoneal inoculation with 1,000,000 cells, mice consistently died after 10-12 days and upon autopsy exhibited typical leukemic spleens with an average weight of 0.4-0.6 g. The spleens of these inbred mice were used as sources of leukemic cells for all the experiments reported here. Akm mice were however used as test animals.

*Tests for Cytotoxic Action of Antisera.* The test dose for all the cytotoxic tests and controls consisted of 100,000 cells introduced intraperitoneally. All antisera had been heated for 30 minutes at 56°C prior to use. Measured amounts of antisera, in the dilutions indicated, were combined with leukemic cells. Guinea pig complement<sup>†</sup> was added in the proportion of 0.1 ml of complement to 1.0 ml of antiserum and cells,\*\* and the mixture incubated

at 37°C for 2 hours with frequent stirring.

*Controls.* The following controls were included: 1) Cells + physiological saline solution, injected without preliminary incubation. 2) Cells + physiological saline solution + complement, incubated for 2 hr at 37°C. 3) Cells + normal rabbit serum + complement.

One series of mice received this mixture immediately while a second series of animals received identical doses after incubation at 37°C for 2 hours. All mice were observed daily and each mouse dying after the 10th day was autopsied and the size of the spleen recorded. At intervals spleens from certain of the groups were combined and the average weight recorded.

*Results. Cytotoxic Tests.* Table I shows the survival rates among 352 mice serving as controls. One of 96 mice receiving the dose of 100,000 leukemic cells immediately after standardization survived. Incubation of the same dose of leukemic cells with complement and physiological saline, or with normal rabbit serum and complement, produced a slight increase in the number of survivals. The differences in survival rates of mice receiving 100,000 leukemic cells in combination with undiluted antisera to normal and leukemic spleen fractions were not significant; therefore, a table is omitted. This confirms Stock's observation with the undiluted antisera to whole cells. There was, however, a significantly lower survival rate of mice receiving antisera to heterologous organs. The spleen lymphocyte thus stimulates the formation of a specific antibody effective against the leukemic cell. When the antisera to the normal and leukemic cell fractions were diluted in serial fashion, prior to combination with the leukemic cells, there was demonstrated a highly significant difference in the cytotoxic action. This suggests that the concentrated antibody of the undiluted sera may have masked the true effects, apparent upon dilution. Table II gives the complete data from titrations.

It is apparent that the mitochondria antisera exhibited the highest titers but the least difference between the normal and leukemic. The  $P_1$  antisera, while of lower titers, exhibited the greatest differences in effects. Compari-

|| The yield of  $P_2$  particles differed quantitatively and it was not possible to obtain sufficient material from the amount of normal spleen used.

10. Arnesen, Kristen, Goldsmith, Yvette, and Dulaney, Anna Denn, *Cancer Research*, 1949, 9, 669.

† Carworth Farms—"Vaeseal."

\*\* It was not determined whether complement was necessary for the action of the serum. This subject is reviewed by Leymaster and Ward.<sup>(11)</sup> It was established however that complement did not interfere in any way with the phenomenon of cell injury.

11. Leymaster, Glen R., and Ward, Thomas G., *J. Immunol.*, 1949, 61, 95.

TABLE I.

Showing the Survivals in the Groups of Mice Serving as Controls. All Received Standard Doses of Leukemic Cells, but No Anti-Sera.

Combination	No. of mice	No. of survivals	% of survivals
Cells and saline; no incubation	96	1	1
Cells and saline and complement; incubated 2 hr	157	8	5
Cells and normal rabbit serum and complement; no incubation	49	2	4
Cells and normal rabbit serum and complement; incubated 2 hr	50	2	4

TABLE II.

Showing the Cytotoxic Action of Diluted Antisera on Leukemic Cells.

Antiserum	Dilution of serum	No. of mice	No. of survivals	% of survivals
Normal mitochondria	1:10	10	9	90
	1:100	10	10	100
	1:200	10	9	90
	1:500*	30	8	27
	1:750	20	3	15
Leukemic mitochondria	1:10	10	10	100
	1:100	10	9	90
	1:200	9	9	100
	1:500*	30	29	97
	1:750	20	12	60
Normal P-1	1:10	30	7	23
	1:100	25	5	20
	1:200†	26	3	12
	1:500	25	2	8
Leukemic P-1	1:10	9	9	100
	1:100	10	10	100
	1:200†	25	24	96
	1:500	24	10	42
Leukemic P-2	1:10	10	10	100
	1:100	10	10	100
	1:200	9	8	89
	1:500	10	0	0
Normal nuclei	1:10‡	34	4	12
	1:50	20	0	0
Leukemic nuclei	1:10‡	34	26	76
	1:50	20	1	5

Serum dilutions used for comparisons are indicated in Table II.

son of the nuclei antisera demonstrated the markedly enhanced cytotoxic effect of the leukemic.

It may be pointed out here that the "infectious property" of the leukemic cells is contained within the cell. A standardized cell suspension was divided and one part stored in the refrigerator, while the other half was washed 3 times with phosphate buffered-

physiological saline solution, and the cells suspended in buffered solution equal to the original volume of physiological saline. One group of mice received the original suspension; the other, the same dose of washed cells. There were no survivors in either group and the time of death was almost identical. No blood or tissue constituent therefore seems necessary for the *in vivo* establishment of the leukemic

TABLE III.  
Showing Results of Challenge Dose of 100,000 Leukemic Cells Given to Groups of Mice Surviving Inoculation of Cell-Serum Mixtures.

Mice surviving after cells + antiserum	No. of mice	No. of survivals	% of survivals
Normal mitochondria*	5	0	0
" P-1	10	0	0
" nuclei	10	0	0
Leukemic mitochondria†	5	0	0
" P-1	10	2	20
" P-2	10	2	20
" nuclei	10	2	20

\* Ten other mice received 10,000 cells. None survived.

† " " " " " " " " " " " "

cell. Furthermore this observation substantiates the use of the term "cytotoxic" in reference to the action of the antiserum. A further observation concerns the question of immunity in mice surviving the antiserum-cell mixture for 45 days. Groups of such mice were given challenge doses of 100,000 leukemic cells. The results of these second inoculations are given in Table III. While the individual groups are small it is apparent that there is a significant difference between the survival rates of the animals "immunized" with leukemic cells exposed to normal antisera, and leukemic cells incubated with leukemic antisera. None of the mice in the first group of 25 survived, while 6 of the 34 making up the second group remained well for the 45 day period.

By use of the point binomial it can be shown that the probability of 6 survivals out of 34 animals is less than 1 in 100,000 if the true death rate is accepted to be 99% (Table I.). Other mice which had also survived the first 45 day period were sacrificed and their spleens examined. Based on macroscopic appearance and actual weight, the spleens would

not be classed as leukemic. Pooled sera of other survivors did not protect mice in experiments employing the standard dose of 100,000 cells.

*Summary.* Antisera to both normal and leukemic spleen cell components exhibit cytotoxic effects for leukemic cells, upon *in vitro* exposure. In every instance the leukemic antisera were much more effective than the corresponding normal antisera. These differences were greater than those obtained by complement fixation procedures. The anti-mitochondria sera exhibited the highest "titers" but the least difference between the normal and leukemic. The most significant differences occurred between the leukemic and normal P<sub>1</sub> antisera, with the leukemic antiserum showing 8 times the activity of the normal. Highly significant differences were also demonstrated between the nuclei antisera. The leukemic antiserum proved over 6 times as effective as the normal. A slight active immunity was demonstrated in mice surviving the combination of leukemic cells and leukemic antisera.

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## Experimental Dental Caries. XIV. Further Studies on Effect of Certain Quinones.\* (17537)

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The investigations on the inhibitory action of certain quinones, with and without vitamin K activity, on acid formation *in vitro*, as well as the contradictory findings on the effect of vitamin K in the prevention of dental caries have been recently reviewed by Dam.(1) In order to study further this problem we(2) carried out an experiment in which was studied the effect of 3 quinones with, and 3 without vitamin K activity, on dental caries activity in hamsters. The results showed that none of the compounds tested induced any significant decrease of dental caries. However, in the discussion of the results obtained we indicated the advisability of testing in a future experiment the effect on caries activity of various quinones and related compounds having different ability to inhibit acid production *in vitro*, and using larger amounts of the compounds than those previously used. It was stated that although in case of obtaining favorable results the implications of such an experiment would not favor the use of quinones, due to their toxicity, in the prevention of dental caries, a study of this kind should indicate how far the ability of certain quinones to inhibit acid production *in vitro* would be paralleled by a corresponding ability of these compounds to decrease caries activity. Therefore the findings thus obtained should contribute to a proper evaluation of the actual relationship between acid production and the development of caries. We are here reporting the results of such an experiment, which was carried out after the inhibitory power of certain quinones on acid production *in vitro* had been determined.

**Experimental.** First, an *in vitro* experiment was carried out in order to determine the inhibitory power of a series of quinones and

related compounds, with and without vitamin K activity, on acid formation by certain oral bacteria. As a source of acidogenic bacteria was used pure cultures of 2 strains of lactobacilli (L<sub>1</sub> and L<sub>2</sub>) and streptococci (S<sub>1</sub> and S<sub>2</sub>) isolated from the deepest portion of a human carious lesion. The substrate used for the growth of streptococci consisted of casein-peptone broth plus 2% glucose. For the lactobacilli was used the same medium supplemented with yeast autolysate. The effect of 13 quinones and related compounds, 6 with and 7 without vitamin K activity, on the growth of the isolated bacteria was tested. 2-methyl-1,4-naphthoquinone (menadione) was used in the amount of 2 mg per 100 cc of substrate; the other substances were tested in the same concentration calculated on a molecular basis. In order to avoid the autoclaving of the compounds to be tested, they were added to the sterilized substrate in the tubes, dissolved either in sterile water or in an easily evaporable organic solvent such as acetone. The tubes were inoculated and then all were incubated with occasional shaking at 37°C for 18 to 20 hours, at which times the pH of the tubes, including the non-inoculated and inoculated controls, was determined with a glass-electrode potentiometer. The results obtained are presented in Table I.

It can be seen from this table that of the quinones derivatives with vitamin K activity methylnaphthoquinone, methylnaphthohydroquinone and methylnaphthohydroquinone disuccinate markedly inhibited acid formation by lactobacilli, and afforded complete inhibition of acid production by streptococci. On the other hand, the tetrasodium and dicalcium salts of methylnaphthohydroquinone diphosphate, as well as the disodium salt of methylnaphthohydroquinone disulfate, all of which have the same vitamin K activity as the first 3 substances, did not inhibit acid production by any of the bacteria tested. This marked inhibitory power of methylnaph-

\* This work was supported by a grant from Rask-Ørsted Fondet.

1. Dam, H., *Vitamins and Hormones*, 1948, VI, 27.

2. Granados, H., Glavind, J., and Dam, H., *Acta Path. et Microbiol. Scand.*, 1949, v26, 597.

TABLE I.  
Comparison of Inhibition by the Compounds Tested of Acid Formation by Lactobacilli and Streptococci.

Kind of acidogenic bacterium	Lactobacillus L <sub>1</sub>	Lactobacillus L <sub>1</sub>	Lactobacillus L <sub>2</sub>	Streptococcus S <sub>1</sub>	Streptococcus S <sub>1</sub>	Streptococcus S <sub>2</sub>
No. of bacteria inoculated per tube (10 cc)	1,200	200,000	400	4,400	20,000	10,000
Incubation time (hr)	20	18	20	20	18	18
pH of non-inoculated tubes	6.95	6.86	6.95	6.89	6.89	6.89
Compounds added to inoculated tubes		pH of inoculated and incubated tubes				
Control	4.70	4.4	4.5	4.2	4.3	4.3
2-methyl-1,4-naphthoquinone (menadiolone)		5.3	6.4	6.9		
2-methyl-1,4-naphthohydroquinone		5.7		6.9		
2-methyl-1,4-naphthohydroquinone diphasphate tetrasodium salt (32.02% H <sub>2</sub> O)		4.6		4.2	4.3	4.3
2-methyl-1,4-naphthohydroquinone diphasphate	4.72	4.6	4.5	4.2	4.3	4.3
2-methyl-1,4-naphthohydroquinone disulfate		4.6		4.2		
2-methyl-1,4-naphthohydroquinone disuccinate						
Benzoquinone	4.75	5.6	6.4	6.9		4.3
Hydroquinone	4.80	4.5	4.7	4.2	4.4	4.3
Quinhydrone	5.41	4.8	4.6	4.2	4.4	4.3
1,2-naphthoquinone	4.69		4.3		4.6	4.4
2-hydroxy-1,4-naphthoquinone	4.50		4.1		5.3	5.1
2,3-dichloro-1,4-naphthoquinone	5.29	4.5	6.4	4.3	6.0	4.8
Anthraquinone-2-sulphonic acid	4.62	4.7	4.5	4.2	4.4	4.3

TABLE II.  
Compounds Added and Caries Activity in the 5 Groups.

Kind and Amt. of each substance added to 100 g of cariogenic diet	Group 1	Group 2	Group 3	Group 4	Group 5
	5 +4	5 +5	5 +5	5 +4	5 +5
	Control	280 mg 2-methyl-1,4-naphthohydroquinone diphosphate dicalcium salt (9.5% H <sub>2</sub> O)	223 mg 2-methyl-1,4-naphthohydroquinone disuccinate	168 mg anthraquinone-2-sulphonic acid	148 mg 2,3-dichloro-1,4-naphthoquinone
Vit. K. activity		+	+	—	—
Inhibits acid production <i>in vitro</i>		—	+	—	+
Av. No. of molars affected	9.6	9.6	9.2	8.4	8.3
Stand. dev.	±0.6	0.3	0.4	0.6	0.1
Av. No. of carious lesions	15.9	15.4	15.5	12.6	13.8
Stand. dev.	±2.0	1.0	2.0	1.6	1.1
Avg caries scores	8.6	8.7	13.4	15.2	7.8
Stand. dev.	±1.1	0.8	4.3	6.2	0.2

thoquinone, methylnaphthohydroquinone and methylnaphthohydroquinone disuccinate on acid production agree with the marked antibacterial effects *in vitro* of the same compounds reported by other investigators.(3,4) Of the 7 compounds with none or negligible vitamin K activity, only dichloronaphthoquinone showed in some cases a considerable inhibitory power on acid production, and hydroxynaphthoquinone inhibited slightly acid formation by streptococci.

Considering the results of these *in vitro* studies, in order to compare the effects of quinone derivatives, with and without vitamin K activity, having marked or none inhibitory action on acid formation), 3. anthraquinone—the following compounds were used in the caries experiments: 1. dicalcium salt of 2-methyl-1,4-naphthohydroquinone diphosphate (with vitamin K activity and without inhibitory action on acid production), 2. 2-methyl-1,4-naphthohydroquinone disuccinate (with vitamin K activity and with marked inhibitory action on acid formation), 3. anthraquinone-2-sulphonic acid without vitamin K activity

and without inhibitory action on acid formation, and 4. 2,3-dichloro-1,4-naphthoquinone (without vitamin K activity and with inhibitory action on acid formation).

Fifty newly weaned hamsters from an inbred colony were littermate distributed into 5 groups (5 males and 5 females in each), and were reared in screen bottom cages without bedding for 100 days on the following basal diet to which was added in each case the quinone derivative indicated in Table II: Salt mixture† 1%, alfalfa meal 2%, Brewer's yeast 5%, powdered whole milk 22%, finely ground yellow corn 25%, and finely powdered sucrose 45%. All the quinone derivatives were supplied in equal molecular concentrations. The groups were given water *ad libitum*, and were weighed weekly. On completion of the 100-day experimental period the animals were sacrificed and autopsied. The molars were prepared for examination and the carious lesions were recorded and scored as previously.(5)

**Results.** At the levels given most of the

3. Armstrong, W. D., Spink, W. W., and Kahnke, J., *Proc. Soc. Exp. Biol. and Med.*, 1943, v53, 230.

4. Atkins, P., and Ward, J. L., *Brit. J. Exp. Path.*, 1945, v26, 120.

† The salt mixture used was McCollum's Salt Mixture No. 185, supplemented with 13.5 mg KI, 139 mg CuSO<sub>4</sub>, 5H<sub>2</sub>O, and 556 mg MnSO<sub>4</sub>, 4H<sub>2</sub>O per 100 g.

5. Granados, H., Glavind, J., and Dam, H., *Acta Path. et Microbiol. Scand.*, 1948, v25, 453.

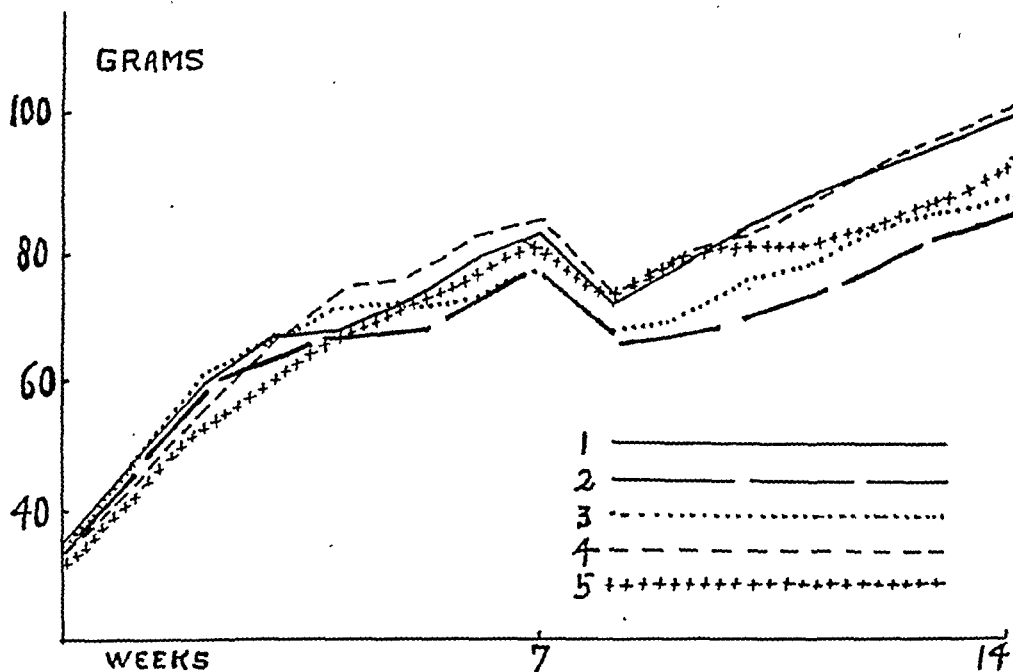


FIG. 1.

Average growth curves of the 5 groups. Group 1, control. Group 2, dicalcium salt of 2-methyl-1,4-naphthohydroquinone diphosphate. Group 3, 2-methyl-1,4-naphthohydroquinone disuccinate. Group 4, anthraquinone-2-sulphonic acid. Group 5, 2,3-dichloro-1,4-naphthoquinone.

quinones derivatives used showed to be toxic through an inhibition of growth. Fig. 1 shows that in increasing order dichloronaphthoquinone (group 5), methylnaphthohydroquinone disuccinate (group 3), and dicalcium salt of methylnaphthohydroquinone diphosphate (group 2) were toxic. On the other hand, anthraquinone sulphonic acid (group 4) did not induce any inhibition of growth as compared with the control (group 1). Table II shows, besides the compounds used, their vitamin K activity and their ability to inhibit acid production, the caries activity of the 5 groups. Beneath the average number of carious molars, carious lesions, and caries scores are presented the standard deviations of the means. The results presented in this table show that none of the quinone derivatives tested decreased the incidence or extent of caries. On the other hand, the higher average caries scores exhibited by groups 3 and 4 are not significant since these increased caries scores were due only to the exceptionally high caries activity of one animal in each of these groups. This is clearly seen from the

much higher standard deviations of the caries scores in these two groups.

**Discussion.** This experiment confirms the negative findings of our previous study on this subject: (2) dicalcium salt of methylnaphthohydroquinone diphosphate and dichloronaphthoquinone were used in both experiments, but in none of them these compounds were able to decrease caries activity, in spite of the fact that in the present study the substances were used in amounts four times higher than those used in the first experiment. Furthermore, although most of the quinone derivatives used in the previous (2) and present studies have shown to inhibit considerably acid formation (6-10) and to have antibac-

6. Fosdick, L. S., Fancher, O. E., and Calandra, J. C., *Science*, 1942, v96, 45.

7. Armstrong, W. D., and Knutson, J. W., *Proc. Soc. Exp. Biol. and Med.*, 1943, v52, 307.

8. Fancher, O. E., Calandra, J. C., and Fosdick, L. S., *J. D. Res.*, 1944, v23, 23.

9. Calandra, J. C., Fancher, O. E., and Fosdick, L. S., *J. D. Res.*, 1944, v23, 31.

terial effects(3-4) *in vitro*, none of them decreased caries activity. Likewise, except dichloronaphthoquinone, all the other substances used in both experiments are readily soluble in water, while methylnaphthoquinone is very slightly soluble. Moreover, of the compounds tested in the present experiment methylnaphthohydroquinone disuccinate deserves special mentioning; it is easily soluble in water, inhibits markedly acid production, and has a definite antibacterial effect.(4) However, in spite of these properties it did not decrease at all the incidence or extent of carious lesions.

Assuming that the quinones tested should have had *in vivo* the same action as they showed *in vitro*, the results of the present studies might be interpreted in various ways. 1. Presuming that the initial lesion in dental caries is primarily due to disintegration of the mineral part of the enamel by acids produced by bacteria in the dental plaque, it could be said that the quinone derivatives tested did not decrease caries activity because they were unable to penetrate the dental plaque, and there exert their inhibitory action on acid formation. However, there have been made only a few studies on the degree of, and factors influencing the penetration of acid-formation inhibiting compounds into the dental plaque *in vivo*. Muntz and Miller(11) have studied the inhibition by certain compounds of the bacterial metabolism of intact dental plaques and of homogenized plaque material, as well as the degree of permeability of the plaques to various substances. They found that the metabolic activity of the intact plaque is inhibited more slowly than that of an equivalent quantity of homogenized plaque material, and that certain very diffusible substances such as urea and glucose penetrated intact plaques slowly. On the other hand, in a previous study on the effect of dietary lactic acid on dental caries activity (given in the food or in the water) we(12) found clear signs of

acid action, combined or not with caries, at the very bottom of the occlusal fossae. This showed that the dietary lactic acid at the concentration given had actually penetrated the intact dental plaques. These facts indicate the complexity of the problem and the need for more fundamental research on the ability to penetrate the intact dental plaque of various substances known to influence the development and/or progress of caries.

2. Another factor which should be considered is that the acid-formation inhibiting power of the quinone derivatives used may have been annulled by the action of some other substances, either of dietary or systemic origin. A 3rd possibility is that the quinone compounds at the levels given, both in the present and in the preceding experiments,(2) may not have encountered in the oral environment sufficient water available for their dissolution and subsequent action. A 4th possibility is that the quinone derivatives which inhibited acid production *in vitro* may have also, at the concentrations given, inhibited acid production in the mouth, inside and outside the dental plaque. In such a case, since there was no decrease of caries activity in any of the groups which were given quinone compounds, this would mean that acid formation should not play any primary role in the development of dental caries since its inhibition would not decrease the incidence of the disease. However, none of the foregoing considerations goes beyond the field of speculation since most of the fundamental processes related to dental caries which take place in the organism, inside and outside the oral cavity, have not yet been adequately and sufficiently studied, and therefore they are not as yet well understood.

Thus, the various studies(6-10) *in vitro* carried out so far, using saliva-glucose mixtures, have demonstrated beyond doubt the acid-formation inhibiting action of a good number of quinone derivatives, with and without vitamin K activity. The first part of the present studies has shown once more such an inhibitory power of certain quinone compounds. Furthermore, the antibiotic effects of various quinone derivatives on a good number of bacteria permanently found in the

10. Fosdick, L. S., and Calandra, J. C., *J. D. Res.*, 1947, v26, 309.

11. Muntz, J. A., and Miller, B. F., *J. D. Res.*, 1943, v22, 73.

12. Granados, H., Glavind, J., and Dam, H., *J. D. Res.*, 1949, v28, 282.



oral cavity have also been demonstrated. (3,4,13,14) On the other hand, the few clinical studies on the effect of 2-methyl-1,4-naphthaquinone incorporated into chewing gum, on caries activity have been contradictory since the decrease in incidence of new carious lesions reported by Burrill and co-workers(15) could not be substantiated by the U. S. Professional Service Schools.(16) Moreover, the previous experimental investigations carried out in rats(17) and hamsters,(2) as well as the present studies, on the effect of quinone derivatives with and without vitamin K activity, in the control of dental caries have all been negative. Therefore, an impartial analysis of the results obtained in clinical and experimental studies on this subject, demonstrate that vitamin K active compounds or other quinone derivatives do not show any promise of controlling caries, and therefore the use of these compounds as a control measure against human dental caries should be discouraged. Certain previous suggestions on the possible beneficial effect of

vitamin K in the control of caries(18) were the product of theoretical considerations which have not been substantiated by actual experiments.

**Summary.** Further studies on the effect of different quinone derivatives with varying degrees of acid-formation inhibiting power, as determined *in vitro*, and with or without vitamin K activity, on dental caries activity were carried out in hamsters. In these studies the following quinone derivatives were tested: Dicalcium salt of methylnaphtho-hydroquinone diphosphate, methylnaphtho-hydroquinone disuccinate, anthraquinone sulphonic acid, and dichloronaphthoquinone. The compounds were used in higher amounts than those used in the previous experiment.

The results showed that none of the quinone derivatives tested exerted beneficial effect against dental caries activity, as compared with the control group. Thus the present experiment confirms the negative findings of previous studies, clinical as well as experimental, on this subject, and indicates that the use of vitamin K active compounds or other quinone derivatives as a control measure against human dental caries should be discouraged. Furthermore, some other implications of these studies have been discussed.

13. Alcalay, W., *Schweiz. Z. f. Path. und Bakt.*, 1947, v10, 229.

14. Kavanagh, F., *J. Bact.*, 1947, v54, 761.

15. Burrill, D. Y., Calandra, J. C., Tilden, E. B., and Fosdick, L. S., *J. D. Res.*, 1945, v24, 273.

16. Professional Service Schools, Medical Department, Washington, D.C., U.S.A., *Bull. U. S. Army Med. Dept.*, 1946, v5, 265.

17. Hatton, E. H., Dodds, A., Hodge, H. C., and Fosdick, L. S., *J. D. Res.*, 1945, v24, 283.

18. Fosdick, L. S., *J. D. Res.*, 1948, v27, 235.

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### The Treatment of *Trichomonas vaginalis* Vaginitis with Aureomycin. (17538)

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Since we(1) had been successful in treating amebiasis with aureomycin it was thought possible that this antibiotic might be useful in other parasitic infestations. It was recalled that many amebicidal preparations are effective

against *T. vaginalis*. Subsequent *in vitro* studies revealed that aureomycin is likewise trichomonocidal. Therefore, it was decided to investigate the action of the local application of aureomycin into the lower female genital tract in *T. vaginalis* vaginitis. A powder for vaginal insufflation was prepared by adding

1. McVay, L. V., Laird, R. L., and Sprunt, D. H., *Science*, 1949, v109, 590.

aureomycin hydrochloride to powdered talc (U.S.P. Merck). The first 6 patients were treated with talc alone. In each of these cases the vagina was insufflated with 2 g of powdered talc on 2 consecutive days and again on the fourth day. At the end of this period the vaginal secretions were negative for *T. vaginalis* in all 6 cases. Three days following cessation of therapy, the vagina contained *T. vaginalis* in 5 of the 6 cases. In the sixth patient vaginal secretions did not become positive until the eighth day. The leukorrhea and vaginal inflammation had persisted.

Aureomycin in dosages of 2 grams a day for 7 days by mouth has been ineffective in the treatment of this condition. Through the cooperation of the Medical, Gynecological, and Obstetrical Services of the John Gaston Hospital 54 cases of *T. vaginalis* vaginitis were obtained for treatment. Twelve of these patients were pregnant and were referred for treatment by the Obstetrical Service. The diagnosis in each case was established by the demonstration of the organism microscopically in the wet-mount preparation.

The preparation for each vaginal insufflation consisted of 500 mg of aureomycin and 2 g of powdered talc. A Holmes insufflator equipped with a rubber cuff and detachable end-piece was employed. The preparation was sprayed evenly over the cervix, vagina, introitus and vulva. Following each treatment patients were instructed to wear a vulvar pad until the next treatment and to refrain from douches or intercourse.

Thirty-one symptomatic non-pregnant patients and 11 asymptomatic non-pregnant patients were insufflated as described above on the first, second, fourth, and sixth days of therapy. Following this the patient was instructed to insert one 250 mg gelatin capsule of aureomycin every other night deep into the vagina for 2 weeks. Twelve symptomatic pregnant patients were similarly insufflated on the first, second, third, fourth, sixth, and eighth days of treatment. Subsequently a 250

mg capsule of aureomycin was inserted each night for 2 weeks. Of the 31 symptomatic, non-pregnant cases there was objective evidence of relapse or reinfection but no return of symptoms in 3 cases. These 3 patients were subsequently retreated successfully. There were no recurrences among the 11 asymptomatic non-pregnant women treated. The rapid subjective and objective improvement of the pregnant cases was most gratifying. These patients were treated more vigorously than the others since it is well established that the disease is much more stubborn to any form of therapy during pregnancy. Three of the 12 pregnant cases showed evidence of reinfection or relapse following therapy, but in only one was there a return of symptoms. This last patient was extremely resistant to treatment and responded only after 3 courses of therapy. All of the 54 patients have been followed at biweekly intervals for 2 to 3 months since stopping treatment.

No significant toxic reactions to intravaginal aureomycin were noted. In 9 of 54 cases mild discomfort, as pruritus, burning or mild intravaginal pain was experienced. This usually occurred after the first or second application of aureomycin and could not always be definitely attributed to the drug. There was no demonstrable adverse effect on pregnancy. It is noteworthy that no objective evidence of local tissue reaction to aureomycin could be demonstrated on physical examination. Moreover, there is no need for concern over any cumulative effects of aureomycin as contrasted to the arsenicals since the small quantities of the drug absorbed from the vagina are rapidly excreted in the urine and bile. Blood levels of aureomycin (done in only 2 cases) 3 hours after vaginal insufflation of 500 mg in talc were in the neighborhood of 0.12  $\mu$ g per cc of blood. Twelve of the cases in this series have been followed by approximately 6 months. In only 2 have trichomonads reappeared.

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oral cavity have also been demonstrated. (3,4,13,14) On the other hand, the few clinical studies on the effect of 2-methyl-1,4-naphthoquinone incorporated into chewing gum, on caries activity have been contradictory since the decrease in incidence of new carious lesions reported by Burrill and co-workers(15) could not be substantiated by the U. S. Professional Service Schools.(16) Moreover, the previous experimental investigations carried out in rats(17) and hamsters,(2) as well as the present studies, on the effect of quinone derivatives with and without vitamin K activity, in the control of dental caries have all been negative. Therefore, an impartial analysis of the results obtained in clinical and experimental studies on this subject, demonstrate that vitamin K active compounds or other quinone derivatives do not show any promise of controlling caries, and therefore the use of these compounds as a control measure against human dental caries should be discouraged. Certain previous suggestions on the possible beneficial effect of

vitamin K in the control of caries(18) were the product of theoretical considerations which have not been substantiated by actual experiments.

**Summary.** Further studies on the effect of different quinone derivatives with varying degrees of acid-formation inhibiting power, as determined *in vitro*, and with or without vitamin K activity, on dental caries activity were carried out in hamsters. In these studies the following quinone derivatives were tested: Dicalcium salt of methylnaphthohydroquinone diphosphate, methylnaphthohydroquinone disuccinate, anthraquinone sulfonic acid, and dichloronaphthoquinone. The compounds were used in higher amounts than those used in the previous experiment.

The results showed that none of the quinone derivatives tested exerted beneficial effect against dental caries activity, as compared with the control group. Thus the present experiment confirms the negative findings of previous studies, clinical as well as experimental, on this subject, and indicates that the use of vitamin K active compounds or other quinone derivatives as a control measure against human dental caries should be discouraged. Furthermore, some other implications of these studies have been discussed.

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### The Treatment of *Trichomonas vaginalis* Vaginitis with Aureomycin. (17538)

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Since we(1) had been successful in treating amebiasis with aureomycin it was thought possible that this antibiotic might be useful in other parasitic infestations. It was recalled that many amebicidal preparations are effective

against *T. vaginalis*. Subsequent *in vitro* studies revealed that aureomycin is likewise trichomonocidal. Therefore, it was decided to investigate the action of the local application of aureomycin into the lower female genital tract in *T. vaginalis* vaginitis. A powder for vaginal insufflation was prepared by adding

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TABLE I.  
Effect of Purified Anterior Pituitary Growth Hormone on Tumor Growth.  
Wt (in g) at autopsy.

Sarcoma 37						Mammary adenocarcinoma					
Controls			Hormone-treated			Controls			Hormone-treated		
3.45	0.56	1.68	2.14	2.29	0.91	2.37	2.55	2.95	1.60	2.30	1.90
3.02	0.22	1.88	2.25	1.69	1.29	2.03	1.55	1.75	1.10	1.15	1.70
2.27	0.55	1.76	2.98	2.90	1.32	1.30	1.30	1.53	2.40	1.10	0.70
1.89	1.07	2.42	2.42	2.13	2.01	1.60	1.35	2.42	1.00	1.28	0.95
1.71	2.58	1.58	1.41	0.29	1.37	1.20	0.45	1.35	1.75	0.47	1.25
1.64	3.57	1.36	1.37	0.41	2.01	1.55	1.40	1.58	1.95	1.43	0.65
2.14	2.67	1.23	2.00	2.83	2.17	0.80	1.10	1.57	1.20	0.63	1.20
1.06	2.14	1.15	1.74	0.76	1.74	0.77	0.70	0.90	1.25	0.72	1.10
0.73	1.92	1.08	2.01	1.48	0.94	0.13	0.60	1.28	1.25	0.49	0.55
0.88	2.02	1.69	1.39	1.12	1.70	0.12	1.40	0.87	0.75	1.03	0.70
1.73 $\pm$ 0.15*			1.70 $\pm$ 0.13			1.35 $\pm$ 0.12			1.19 $\pm$ 0.095		

\* Mean values  $\pm$  standard error.

(Armour). The mice receiving growth hormone showed a marked increase in weight during the first week of therapy. After this time, when the tumors were becoming necrotic, all groups suffered weight losses.

The animals were killed by decapitation; the tumor was peeled away from the subcutaneous connective tissue of the host, the interior exposed, and all visible necrotic material removed. The weights of the tumors are recorded in Table I.

It can be seen that the purified growth hormone did not show any appreciable effect, stimulatory or inhibitory, on the transplant-

able tumors used. Histological studies, which were kindly performed by Dr. E. Lowenhaupt of the Department of Pathology, revealed that tumors of all groups of animals, hormone-treated and controls, were of essentially similar appearance as regards relative proportions of necrotic tissue, numbers of mitotic figures and cell appearance.

*Conclusion.* Purified growth hormone does not influence the growth of sarcoma 37 and the mammary adenocarcinoma in "A" strain mice.

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## Activation of Purified Prothrombin.\* (17540)

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The activation of prothrombin has probably been discussed more extensively than any other problem in blood coagulation. It is an important process and exceedingly complex in nature. Calcium, thromboplastin, Ac-globulin and platelet derivatives participate in this vital function; and although it is not exactly clear how the activators work, it is apparent that they may be divided into two main classes; namely, those with thromboplastin activity in the traditional sense and those with accelerator

or thromboplastin co-factor activity. Either class alone is a poor activator of purified prothrombin, but together they cause rapid conversion. To understand more exactly what forces these substances bring to bear on prothrombin it is essential that they be obtained in purified form and in sufficient quantity for study. This still needs to be accomplished, but in the meantime it may be possible to obtain indirect information concerning the mechanism of their action. For example, this

# Effect of Purified Growth Hormone on Tumor Growth.\* (17539)

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Alterations of hormonal processes have been suspected as being involved etiologically in neoplastic diseases. Several early experiments(1,2) indicate that anterior pituitary extracts stimulated the growth of inoculated tumors, while conflicting findings exist regarding the therapeutic success of similar extracts in inoperable cancer patients.(3,4) Stern(5) believes that "the anterior pituitary secretes two groups of hormones which are antagonistic as to their effect on tumor growth." Extracts containing the growth hormone principle are thought to stimulate neoplastic growth(6,7) while gonadotropic factors seem to inhibit tumor growth.(8,9) These divergent views may be attributed to differences in the experimental tumor and species of animal employed, as well as to the variable nature of the endocrine preparation used. This study was prompted by the recent availability of purified growth hormone (Armour<sup>†</sup>) in response to a new method of preparation,(10) and was undertaken to determine whether tumor growth could be influenced by the hormone. The activation of normal growth

under the influence of the anterior hypophyseal hormone is well-known.(11,12)

Inbred "A" strain mice of both sexes were used. The animals weighed between 18 and 24 g, and were 2 to 3 months old. The mice received a diet of commercial pellets (Purina Lab Chow), weekly supplements of fresh lettuce, and were permitted access to food and water at all times. Tumors were transplanted by the trocar method, each animal receiving a fragment of neoplastic tissue in the right inguinal region. Sarcoma 37 and the familiar mammary adenocarcinoma were the test tumors. The latter tissue was not as satisfactory as the sarcoma, since there was the occurrence of late visceral metastases,(13) a tendency towards the formation of cysts containing white stringy material, and an earlier onset of visible necrosis than in the transplantable sarcoma.

Six days after tumor transplantation, the hormone therapy began. The animals included for the experiment had tumors of the following dimensions,  $3.5 \pm 1.5 \text{ mm} \times 3.0 \pm 1.0 \text{ mm} \times 2.5 \pm 0.5 \text{ mm}$ . For the first two days, the daily dose was 0.1 mg growth hormone<sup>‡</sup> in 0.2 ml distilled water (pH adjusted to 7.4 with sodium bicarbonate); from the third to the tenth day, the daily dose was 0.2 mg; the dose was increased to 0.5 mg on the eleventh day and continued at this level for 4 more days at which time the experiment was terminated. Throughout the experiment, the controls received corresponding amounts of crystalline bovine plasma albumin

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‡ A preliminary assay on plateaued female Long-Evans rats (about 200 g) showed that as little as 0.5 mg of the hormone caused a net increase of 10 g in weight over the controls in a 24-hour period.

No attempt was made to have the concentration of the sulfones equal on a molecular weight basis.

**Prothrombin Activation.** Powdered sodium citrate was added to an aqueous solution of prothrombin so that the concentration of sodium citrate was 25% and the concentration of prothrombin was about 1.5%. Then the crystalline sulfone was added in sufficient quantity so that crystals could be seen suspended in the prothrombin solution. The mixture was allowed to stand at room temperature, and samples were taken for thrombin analyses. The results presented in Fig. 1 were obtained with the same prothrombin preparation and, were obtained simultaneously so as to obtain the best possible comparable results with the various sulfones.

**Results.** With the 25% sodium citrate solution prothrombin activation was slow during the first 5 hours. After this induction period activation was rapid, and within a day the maximum yield of thrombin was obtained. This was, however, only 67% of the potential thrombin which could be obtained from the prothrombin with the use of calcium, thromboplastin and Ac-globulin. The thrombin titer was followed for 3 more days and showed a progressive decline similar to that commonly encountered with thrombin solutions which are not stabilized with glycerol or carbohydrates. (6) By adding 3, 4, 4'-triaminodiphenyl sulfone, or 2-hydroxy-4, 4'-diaminodiphenyl sulfone to the prothrombin in 25% sodium citrate solution the induction period was prolonged to more than a day and even thereafter only small amounts of thrombin formed so that in 5 days the thrombin yield was less than 25%. These compounds can thus be considered powerful inhibitors of prothrombin activation under the conditions of the experiment.

It is also possible to obtain the opposite effect; namely, an acceleration of prothrombin activation. The most interesting compound studied was 3-chloro-4, 4'-diaminodiphenyl sulfone. It prolonged the induction period about 2 hours. Then thrombin formation was far more rapid than with the citrate

control and the final yield was almost 100% as compared with 67% for the control. The deterioration of the thrombin solution was at a rate typical of other thrombin solutions. Several other compounds were also found to be accelerators. Two of these, 4, 4'-diaminodiphenyl sulfoxide and 3-sulfonamide-4, 4'-diaminodiphenyl sulfone doubled the induction period but once thrombin began to form it continued until a high yield was obtained on the third day. Two others, 2, 4, 4'-triaminodiphenyl sulfone, and 4, 4'-diaminodiphenyl sulfone prolonged the induction period to one day. The thrombin titer then rose very slowly so that a 67% yield was obtained on about the fourth day.

**Discussion.** There are two practical benefits which can be derived from these experiments. (1) The preparation of thrombin from prothrombin is simplified. By adding 3-chloro-4, 4'-diaminodiphenyl sulfone to purified prothrombin in 25% sodium citrate the yield is increased to a maximum. There is thus no loss of an expensive prothrombin product, or remnants of proenzyme with the desired thrombin. (2) The preparation of prothrombin is simplified. In the purification procedures it has been difficult to eliminate thrombin from the final product. Failure to do so causes the prothrombin to be altered by the small amounts of thrombin. (7) This hazard can now be eliminated, for the ammonium sulfate used for fractionation work, like sodium citrate, causes slow activation of prothrombin, and this effect of ammonium sulfate can be blocked by adding small amounts of 3, 4, 4'-triaminodiphenyl sulfone to the ammonium sulfate solutions used in the fractionation procedures.

It has been discussed repeatedly that thromboplastin and Ac-globulin together activate prothrombin more rapidly and give a higher yield of thrombin than when the former is not supplemented with Ac-globulin. This general observation also applies to these artificial activators. Sodium citrate alone, like thromboplastin, acts slowly and the yield of thrombin is incomplete, but sodium citrate with 3-

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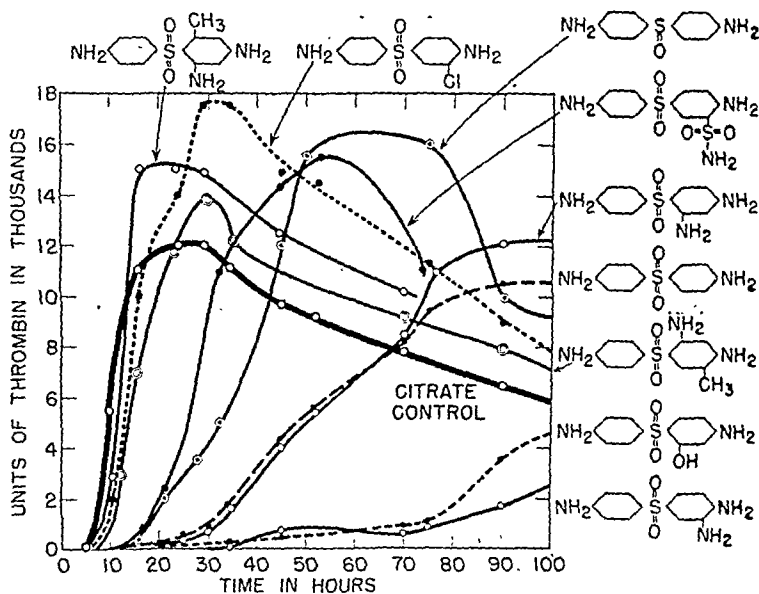


FIG. 1.

Activation of purified prothrombin in 25% sodium citrate solution, and of the same prothrombin in 25% sodium citrate solution together with a saturated solution of the organic compound indicated for each curve. The concentration of prothrombin was 18,000 units per cc.

paper describes prothrombin activation experiments in which sodium citrate, an organic compound of known structure, is the activating agent. This activation can be either blocked or synergized by certain diphenyl sulfones whose structure is also known. It should be possible to elucidate the nature of the forces required for prothrombin conversion by studying the action of these relatively simple compounds as activators and inhibitors, and thus by analogy formulate viewpoints concerning the more complex physiological activators. The work to be described is sufficiently promising to justify a hopeful outlook for the new attack on the problem of thrombin formation.

**Experimental. Thrombin Analysis.** Quantitative measurement of thrombin activity was done by the method of Seegers and Smith.<sup>(1)</sup>

**Prothrombin.** Purified prothrombin was obtained from bovine plasma by methods pre-

viously described.<sup>(2,3)</sup> Only high quality material was used. Generally the specific activity was 1200 units per milligram dry weight or more. It was free of Ac-globulin activity and contained no thrombin. On electrophoresis such products have a main boundary pattern comprising about 90% of the total. Quantitative determination of activity was by the 2-stage method.<sup>(4,5)</sup>

**Diphenyl Sulfones.** These were all synthesized in the laboratory and it is expected that the method of synthesis will be described in detail elsewhere. I wish to thank Leonard Doub for placing these compounds at my disposal. The crystalline compounds were added to the prothrombin dissolved in 25% sodium citrate solution. All are sparingly soluble and it required only a few milligrams to give a saturated solution with respect to the sulfone.

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TABLE I.  
50% Endpoints of Virus Titrations Done in:

Exp. No.	(a) 10-11-day eggs allantoically	(b) in 15-day "empty" eggs	(c) difference in logs
1	10-7.46	10-10.00	2.54
2	10-7.57	10-9.0	1.43
3	10-7.33	10-8.57	1.24
4	10-7.66	10-8.71	1.05
		Avg diff.	1.57

scissors. The chorio-allantoic membrane which is firmly attached to the shell membrane is the only structure remaining in the egg. The internal surface of the membrane is now thoroughly washed with 3 changes of cold 0.85% NaCl so as to remove any remaining yolk, albumen, and blood. After the egg is drained by placing it with the open end down on a sterile petri dish, it is filled with 10-40 cc of Tyrode's solution containing virus, and 10 units of penicillin and 40  $\mu$ g of streptomycin per cc. The opening is closed with a sterile rubber cap, such as is used for centrifuge tubes, which is lined and then sealed to the shell with hot paraffin. The eggs in suitable trays are then put in a test tube roller which is kept in an incubator at 37° and makes six revolutions per hour. Fluid can be withdrawn from the eggs at desired intervals by piercing the rubber cap with a sterile needle attached to a syringe.

It has also been found possible to infect eggs first allantoically in the regular way and after the virus has been adsorbed to the membrane or as late as 48 hours after infection the contents of the egg are poured out. The interior is washed thoroughly and filled with Tyrode's solution. After further incubation at 37° virus increase can be demonstrated.

**Results.** Thus far, the PR8 strain of influenza virus has been used in the experiments. Hemagglutination titers of 1280-2560 in a pattern test were regularly obtained in the fluid after 24-hours' incubation when a 10-20 ml volume of  $10^{-5}$  to  $10^{-6}$  dilution of virus was used as inoculum. Under the same conditions a hemagglutination titer of 5120 is usually obtained with allantoic fluid of the complete egg infected with the PR8 strain. The specificity of the reaction is demonstrated by the inhibition obtained with specific serum against in-

fluenza virus, Type A. It is obvious, therefore, that the chorioallantoic membrane produces virus under the described conditions in much the same amounts as in the intact egg. Several tests were done in which the sensitivity of this method for detecting small amounts of virus was compared with that of allantoic inoculation. Ten-fold dilutions of allantoic fluid infected with PR8 strain were prepared in Tyrode's solution and inoculated in 0.1 ml amounts in 10-11 day old eggs allantoically and in 20 ml amounts in egg cavities prepared by the method described. Four eggs were used per virus dilution. The allantoic inoculations

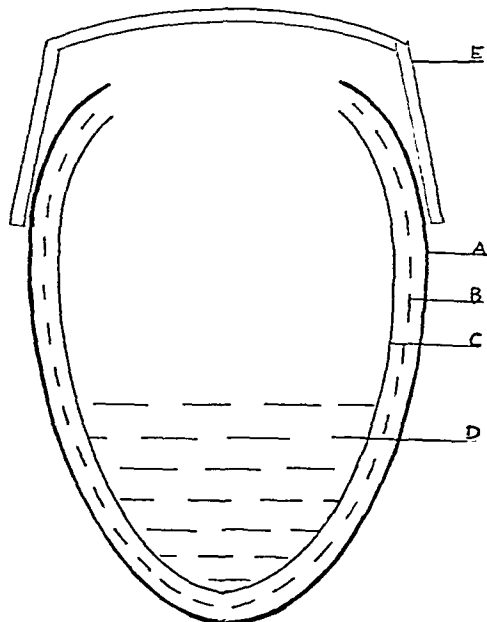


FIG. 1.

A. Egg shell. B. Shell membrane. C. Chorio-allantoic membrane. D. Tyrode's solution. E. Rubber cap.

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chloro-4, 4'-diaminodiphenyl sulfone, like thromboplastin with Ac-globulin, activates prothrombin more rapidly and the yield of thrombin is increased. The similarity between the action of the artificial activators and the physiological ones would seem to be of more than coincidental significance, even though the time intervals involve hours in one instance and minutes in the other.

Perhaps the most remarkable result of these experiments is the difference in action of 3, 4, 4'-triaminodiphenyl sulfone and 3-chloro-4, 4'-diaminodiphenyl sulfone. One has an amino group in the three position and the other a chlorine atom. One is an inhibitor and the other an accelerator. Thus, for one set of conditions, the difference between a "coagulant" and an "anticoagulant" has been reduced to a locus on the benzene ring where

placement of an amino group produces one effect and a chlorine atom the opposite. This subtle detail is easily brought into focus, and perhaps it is here where we can hope to see, for the first time, what forces physiological anticoagulants and coagulants bring to bear on prothrombin.

*Summary.* Purified prothrombin can be activated by dissolving it in a 25 per cent solution of sodium citrate, but the yield of thrombin is usually low. By adding a small amount of 3-chloro-4, 4'-diaminodiphenyl sulfone to the activation mixture the yield of thrombin is increased to the maximum. Several other diphenyl sulfones also increase the yield. In contrast, 3, 4, 4'-triaminodiphenyl sulfone and 2-hydroxy-4, 4'-diaminodiphenyl sulfone act as inhibitors of the activation process.

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### Cultivation of Influenza Virus in the Chorio-Allantoic Membrane of Deembryonated Eggs.\* (17541)

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Fertilized chicken eggs have proved to be an excellent medium for the growth of many viruses and other pathogenic agents. A number of viruses grow in the various tissues of the embryo and in the membranes enveloping it in an indiscriminate manner while others show specific affinities for certain structures in the egg. For a number of viruses, among them those of the influenza group, the chorio-allantoic membrane(1-3) is the main site of virus multiplication. Most other structures

offer less favorable conditions. It seemed an obvious step, therefore, to use only the chorio-allantoic membrane *in situ* for virus growth and to eliminate the other constituents of the egg. For this purpose the following technic was found practicable.

*Methods.* Eggs which have been incubated for 14-15 days are candled and the outline of the air-sac is marked. One-half centimeter above this line the egg shell is cut with an electric drill and the piece of shell is removed. The free-lying shell membrane and the marginal zone of the shell both on the inside and outside along this opening are covered with hot paraffin. The shell membrane together with the piece of underlying chorio-allantoic membrane is then cut away with a pair of curved scissors. By gently tilting and rotating the egg the embryo and the yolk sac are poured out and their connections with the chorio-allantoic membrane are severed with

\* This investigation was conducted under the auspices of the Commission on Influenza, Armed Forces Epidemiological Board, Office of the Surgeon General, U. S. Army, Washington, D.C.

† Aided by a fellowship from the Dazian Foundation for Medical Research.

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pre-existing renal damage. It was determined soon after their introduction that mercurial diuretics acted directly upon the kidney, (9-12) and there has been general agreement that diuresis results from depression of tubular function. The localization of this action in the tubule has been studied more recently, but has not been completely clarified. There are some indications that the effect is produced upon the proximal tubule, (13,14) whereas some evidence indicates an effect upon the distal tubule. (15) To further complicate matters, there is evidence for species differences in the effects of mercurial diuretics. (13,16)

Because it seemed probable that mercurial diuretics are potentially toxic substances which act by depressing tubular function, it seemed remarkable that such toxicity does not become clinically evident in patients with the nephrotic syndrome, when mercurial diuretics are used. Here, in a situation which is accompanied by tubular degeneration, it would seem reasonable to suppose that damaged tubule cells, seen as desquamated cells in the urinary sediment, would be more susceptible than normal cells to the toxic action of these compounds. Yet such patients often receive mercurial diuretics without ill effect, though often, in our clinical experience, without therapeutic effect. As a result of such observations and reflection, we became interested in studying the effect of proteinuria upon the toxic action of mercurial compounds.

TABLE I.  
Effect of Proteinuria on Kidney Weight and Body Weight After Administration of Mercurial Diuretic.

Group No.	IP inject.	No. rats	Mercurial used	Mercurial inj. after IP	No. rats surviving	Time kill interval survivors, days	Initial wt, g	Final wt, g	Kidney wt, mg	Anatomic diagnosis
1	Saline	6	Mersalyl	111	6	1	153		1137	Nephrosis, acute, very slight
1a	Albumin	6	"	111	6	1	154		1226	Nephrosis, acute, very slight
2	Saline	12	"	111	12	1	158		1103	Nephrosis, necrotizing, acute, severe
2a	Albumin	12	"	111	12	1	159		1273	Nephrosis, acute, slight to moderate
3	Saline	14	Mercalur.	111	10	7	153	134	1635	Nephrosis, acute, severe, advanced healing
3a	Albumin	13	"	111	13	7	152	155	1183	Nephrosis, acute, slight
3b	"	6	"	11	6	7	154	154	1167	Nephrosis, acute, very slight
3c	"	6	"	1	1	7	150		2561*	Nephrosis, acute, severe, healing, slight hydropnephrosis

\* One surviving animal only.

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were always performed first. After 48 hours' incubation at 37°C all fluids were tested for hemagglutinins. Table I shows the results of several experiments in which the 50% end-points of infectivity obtained with the two methods were calculated by the method of Reed and Muench.(4) It is seen that the titers of allantoic fluid as measured by allantoic sac inoculation were always lower than those obtained with the prepared membrane alone. Since a much larger amount of fluid is introduced into the "empty" eggs, a correspondingly higher amount of virus is also inoculated. This may explain the greater sensitivity observed. Other explanations seem possible.

**Discussion.** The method may have various applications. It is possible to introduce larger amounts of fluid into the egg than by other methods and this may be of advantage when virus isolation from throat washings are

undertaken. The influence of fluids of different composition on virus multiplication and the effect of inhibitors and accelerators of growth can easily be studied by this method which allows virus growth in a practically intact tissue. Moreover, the rate and character of growth of virus in the membrane may be more accurately studied. Such experiments are in progress. The method may have certain advantages for the production of vaccines as virus multiplication can be obtained in a medium of chosen composition, and the presence of urates in the virus fluid is excluded.

**Summary.** A method is described in which the chorioallantoic membrane of the egg *in situ* is used for the cultivation of influenza virus. The sensitivity of the method is demonstrated and various possibilities for its application are discussed.

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## Effect of Proteinuria on Toxicity of Mercurial Diuretics in the Rat. (17542)

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During the past 30 years, organic mercurial compounds have been used widely for the purpose of inducing diuresis. Because of their apparently low clinical toxicity, mercurial diuretics have been used with impunity in the presence of cardiac failure, and, even in the presence of impaired renal function, few clinicians have hesitated to administer them. Studies in animals(1-4) gave results which, while in some degree conflicting,

nevertheless indicated that the agents could produce transient or permanent renal damage in doses which were comparable to those recommended for use in man. When clinical application of the mercurial diuretics was begun, it soon became evident that, although there was some indication of renal irritation,(4-6) the clinical toxicity was negligible,(7,8) at least in the absence of severe,

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animals died from the fourth to the sixth day after injection. At the end of a week, the survivors were severely ill, emaciated, and hyperirritable when disturbed. In this group the kidneys were greatly enlarged, with extremely pale granular surfaces. On sagittal section, the cortex was very pale and striated, while the medulla appeared to be relatively well preserved and of almost normal color. On microscopic examination, the tubular structure was disrupted, with appearance of severe toxic nephrosis in an advanced healing stage. (Fig. 3, 4). In sharp contrast, the animals in Group 3a (albumin + 1.95 mg Hg) appeared to be entirely normal until



Fig. 3.

Kidneys, gross specimens. Left: Group 3, saline-injected animals, 7 days after meralluride sodium (1.95 mg Hg). Right: Group 3a, albumin-injected animals, same interval and same dose of meralluride sodium, injected after albumin injection No. III. Note difference in size and color of kidneys.

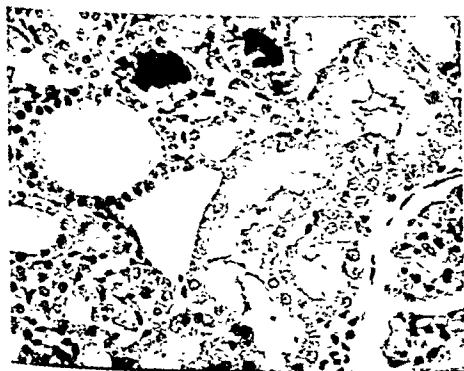


Fig. 4.

Section of kidney, Group 3: saline-injected animals, 7 days after meralluride sodium (1.95 mg Hg), showing necrotizing nephrosis with healing and deposits of calcific material. Hematoxylin-eosin stain, 240X.

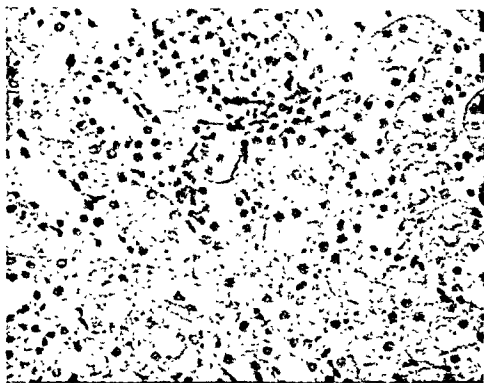


Fig. 5.

Section of kidney, Group 3a: albumin-injected animals, 7 days after meralluride sodium (1.95 mg Hg), administered at time of albumin injection No. III. Note relatively intact tubular structure with mild changes of acute nephrosis. Hematoxylin-eosin stain, 240X.

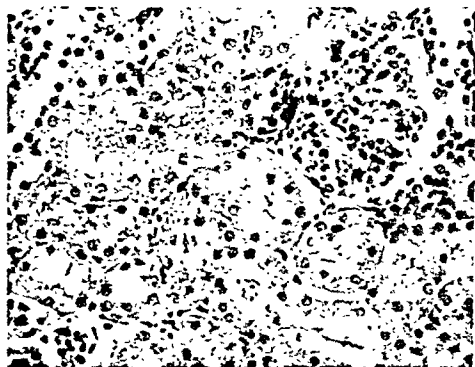


Fig. 6.

Section of kidney, Group 3b: albumin-injected animals, 7 days after meralluride sodium (1.95 mg Hg), administered at time of albumin injection No. II. Note that degree of damage is relatively slight, similar to that in Fig. 5. Hematoxylin-eosin stain, 240X.

killed. All survived in good health and there was a slight gain in weight. At autopsy, the kidneys were normal in size and appearance except for the slight pallor mentioned under Group 2a. Microscopically there was a very slight degree of tubular damage (Fig. 5). In Group 3b (albumin + 1.95 mg Hg after inject. No. II) the animals were entirely comparable to those in Group 3a, and the kidneys were virtually indistinguishable (Fig. 6). The animals in Group 3c (albumin + 1.95 mg Hg after inject. No. I) were the most severely affected of all. Only one animal survived the

**Methods and materials.** This study was performed with 75 female rats, each weighing about 150 g. The organic mercurial diuretics studied were mersalyl-theophylline and meralluride sodium. Mersalyl-theophylline contains 39.6 mg Hg and 50 mg theophylline in 1 ml. Meralluride sodium contains 39.0 mg Hg and 48 mg theophylline in 1 ml. In the dosages tabulated (Table I) theophylline was present in the above ratio to the dose of Hg. The animals each received three 16 ml intraperitoneal injections, No. I at 9:30 A.M. and No. II at 4:30 P.M. on the first day, and No. III at 9:30 A.M. on the second day. For the control groups, the injection material was 0.85% sodium chloride solution. For the experimental animals, the injection material was 6% human albumin in 0.85% sodium chloride solution. Immediately after the designated intraperitoneal injection (Table I), the mercurial diuretic was administered intravenously, diluted to give the proper dose with 0.85% sodium chloride solution. The animals were then left undisturbed upon stock diet either until death, or for the interval specified, at which time they were killed by ether anesthesia and exsanguination from the abdominal aorta. The kidneys were removed promptly, except in the few animals that died during the night, decapsulated, sagittally sectioned, blotted, weighed, and dropped into 10% formalin. After fixing, paraffin sections were made and stained with hematoxylin and eosin for microscopic examination.

**Results.** The animals in Group 1 (saline + 0.26 mg Hg) and 1a (albumin + 0.26 mg Hg) appeared perfectly normal until killed. At autopsy they also appeared perfectly normal. There was no appreciable difference between the groups in kidney weight, except for the difference expected after albumin administration. The kidneys appeared normal in the gross, both externally and on sagittal section. In the microscopic sections there were minimal changes of acute nephrosis present in both groups. There was no convincing difference in the degree of nephrosis between the two groups. The animals in Group 2 (saline + 0.79 mg Hg) and 2a (albumin + 0.79 mg Hg) also appeared normal until killed. At autopsy, as in all the animals, there were no significant findings

outside of the kidneys. In Group 2 the kidneys were somewhat smaller than normal, with slight pallor of the surface and the cortex. In Group 2a the kidneys were normal in size and the pallor was hardly evident, resembling the slight pallor that accompanies protein injections alone, in our previous experience. Microscopically, the kidneys in Group 2 showed definite and pronounced tubular damage (Fig. 1), while those in Group 2a showed slight tubular damage of a much milder degree (Fig. 2).

In the animals of Group 3 (saline + 1.95 mg Hg), the findings were entirely different. These animals were obviously ill from the day of the mercurial injection and 4 of the 14

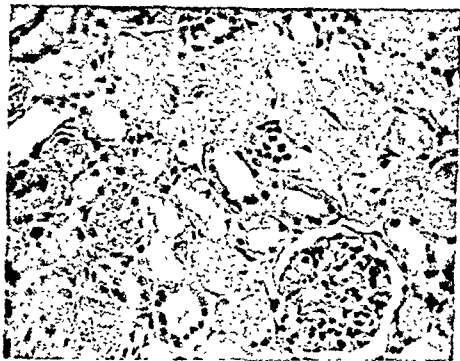


FIG. 1.  
Section of kidney, Group 2: saline-injected animals, 1 day after mersalyl (0.79 mg Hg), showing acute necrotizing nephrosis. Hematoxylin-eosin stain, 240X.

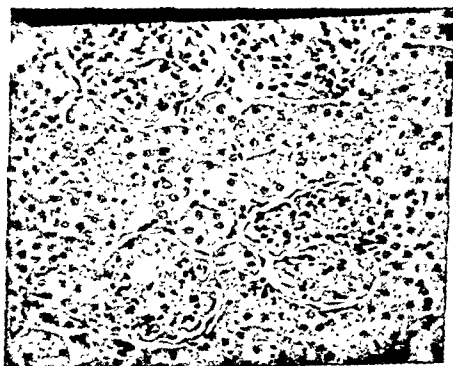


FIG. 2.  
Section of kidney, Group 2a: albumin-injected animals, 1 day after mersalyl (0.79 mg Hg), relatively free of tubular damage. Hematoxylin-eosin stain, 240X.

pected relative innocuousness and therapeutic ineffectiveness of mercurial diuretics in patients with renal disease may be related to the presence of marked proteinuria.

*Addendum.* After submission of this paper, we became aware of work done by Havill, Lichty, and Whipple,(21) showing that the tolerance of dogs for mercury bichloride was increased by frequent hemoglobin injections. It seems reasonable to assume that the mechanism of protection from the toxic action of the metallic ion was similar in this instance, since hemoglobin, like albumin, is reabsorbed by the proximal tubule cells until saturation of the reabsorptive capacity occurs, although Havill and his co-workers did not relate the protective effect to the occurrence of hemoglobinuria, which they did not measure, suggesting instead that the pigmented moiety of the hemoglobin molecule was responsible for the protection.

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*Summary.* Administration of human albumin to rats, with resultant proteinuria, prior to administration of mercurial diuretics, sharply reduces renal toxicity of the mercurial. This effect is attributed to inhibition of mercurial reabsorption when the tubules are saturated with protein.

Simultaneous administration of human albumin and the mercurial enhances toxicity. This effect is attributed to the more rapid absorption of mercurial, concomitant with more rapid reabsorption of protein during the tubular loading phase.

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## Fetal Death and Maldevelopment Resulting from Maternal Vitamin A Deficiency in the Rat.\* (17543)

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The course of pregnancy may be severely altered by feeding female rats a diet restricted in vitamin A, the severity of the alteration being roughly proportional to the degree of vitamin deprivation. Death and resorption of the fetuses, prolongation of pregnancy, and delivery of stillborn young were reported by Mason(1) and Cannon(2) who employed deficient diets with little or no supplement of vitamin A or carotene. Warkany and Schraffenberger(3,4) using similar diets supplemented with small amounts of carotene also

noted a high incidence of fetal resorption; but, by interrupting pregnancy at various times from the 13th day to term, they were able to obtain a number of living and recently dead fetuses and newborns suitable for histologic study. The eyes of these offspring were found to bear several developmental anomalies, notably: folding and eversion of the retina, persistence of the choroidal fissure, absence of the ciliary body, and postlenticular fibroplasia. Thus, deficiency of vitamin A in the diet of female rats prior to and during pregnancy not only reduces fecundity by

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full week, and it appeared moribund. The kidneys of those dying earlier showed pronounced enlargement, but those of the one surviving were most remarkably enlarged, almost chalky white, with a granular surface. On sagittal section, the entire kidney was similarly without color in both medulla and cortex. Microscopically, the tubular damage was profound and more severe than in any other group (Fig. 7).

**Discussion.** The data given here show conclusively that intraperitoneal administration of albumin to rats, in large doses, prior to the administration of a severely toxic dose of mercurial diuretic, effectively protects the kidneys from the toxic effects of mercury. On the contrary, when the protein is administered simultaneously with the mercurial, the toxic effect of mercury is enhanced. Many previous experiments with administration of protein, in the same dosage used in this study, have established the relation of proteinuria to the time of injections.(17,18) The first appearance of proteinuria after such injections has been interpreted as indicating that the maximum tubular capacity for reabsorbing protein has been exceeded, so that the rate of protein reabsorption is critically diminished. This

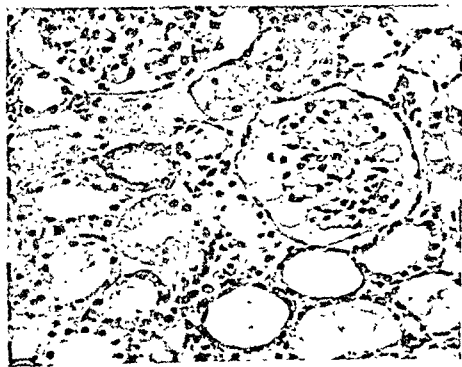


FIG. 7.

Section of kidney, Group 3c: albumin-injected animals, 7 days after meralluride sodium (1.95 mg Hg), administered at the time of albumin injection No. 1. Note extreme severity of toxic nephrosis. Hematoxylin-eosin stain, 240X.

coincides with the time of our injection No. II. Therefore, it seems probable that the diminished toxicity of mercurial diuretic at this time is associated with diminished tubular reabsorption of mercury as well as protein. On the other hand, it seems plausible to suppose that, during the interval from the first protein injection to the first appearance of proteinuria, the reabsorption of protein proceeds at a more rapid rate than normal, and that during this period the reabsorption of mercury is similarly increased, so that the toxicity of a given intravenous dose is enhanced. That mercurial diuretic is selectively concentrated in the kidney, in patients who have received mercurial diuretics(19) and in normal dogs,(20) is already known. Little is known about the precise molecular structure and physical properties of the mercurial diuretics. The prosthetic, mercury-containing portion of the complex is unstable except in combination with theophylline. However, the precise nature of the combination is not known, nor is the size of the complex known. We have tried to dialyze meralluride sodium, and found that after 48 hours virtually none had passed through a cellophane membrane. In an effort to ascertain whether the mercurial diuretics are bound by serum protein, meralluride sodium was incubated with 6% human albumin and then separation was attempted by precipitating the protein, using trichloroacetic acid, phosphotungstic acid, and phosphomolybdic acid. In each case, the meralluride sodium did not appear to be bound by protein, all remaining in the supernatant fluid. Therefore, it would appear that inhibition of mercury reabsorption is probably not related to a union between the mercurial diuretic and protein, as such. Experiments are in progress to determine by cytological demonstration whether our hypothesis, that proteinuria reduces the quantity of mercury reabsorbed, is correct.

From these findings, it is suggested that our clinical experience concerning the unex-

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TABLE I.  
Course and Termination of Pregnancy in Vitamin A Deficient and Control Rats.

Age of fetuses when removed or delivered, days	Days of pregnancy on which untoward signs were observed			Condition of young		
	Cornified cells in smear (25% or more)	Abnormal blood in smear	Wt loss by mother	No. dead or resorb.	No. living	Mal- formed
22	—	—	19	0	11	No
22	—	—	—	2	7	Yes
22	—	—	11	2	8	Yes
20	6-16, 18, 20	18, 20	14	all		
19	7-14, 17, 18	10, 11, 16, 19	—	3	6	Yes
19	—	19	14	4	8	Yes
19	19	15, 17, 18	15, 18, 19	all		
19	7-18	10, 11, 19	15, 16	all		
18	4-11, 13, 14, 17, 18	15, 17, 18	13	all		
17	—	15, 16, 17	12, 13, 15	7	5	Yes
16	13-16	15	12, 14	all		
16	8, 10, 11	11, 15	14, 15, 16	all		
15	8-15	15	—	all		
15	1-13	11	14, 15	all		
15	9-11, 13	15	11	all		
15	4, 9, 14	15	13, 15	5	5	Yes
Controls						
22	—	—	12	3	9	No
22	—	15, 16	—	0	3	No
22	13, 14	17-20	13	4	4	No
22	—	—	—	0	14	No
22	—	—	—	0	7	No
20	—	—	—	1	13	No
17	—	—	13	0	11	No

by the mother) the future course of pregnancy might be determined with some degree of accuracy.

*Fetal death.* Of the 36 females raised until maturity on the preparatory diet 34 mated as indicated by sperm in the vaginal smear, but only 30 were later verified to have become pregnant. The first 16 pregnancies, after being transferred to the more highly purified diet, were allowed to continue to term or until there was definite evidence of prior resorption of some of the fetuses. Thus, this group provided a check on the reliability of the proposed criteria to be used in predicting intra-uterine destruction of the fetuses. Only 3 of these 16 pregnancies were sufficiently uneventful that they were allowed to proceed to the 22nd day of gestation (Table I). Thirteen were interrupted earlier by killing the mother when it was judged unlikely that any of the conceptuses would survive until term. Such judgment was made after at least two, and usually all, of the following signs became positive: 1) 25% or more of cornified cells in the vaginal

smear during pregnancy; 2) blood in the vaginal smear in excess of, or at times other than, that characteristic of the "placental sign", which appears normally as a trace between the 11th and 15th days; and 3) a loss of weight by the mother, particularly after the 10th day.

It is apparent from the data in Table I that these criteria were of value in indicating the fate of the fetuses. In the absence of cornified cells and abnormal quantities of blood in the vaginal smear, a majority of the fetuses may be expected to survive until the 22nd day, as was observed in 3 vitamin A deficient and a large percentage of the control mothers. Conversely, cornified cells, to the extent of 25% or more were observed at one time or another in 11 of 13 females whose pregnancy was later interrupted. All fetuses in 9 and several in the remaining 2 pregnancies were dead or already undergoing resorption. Two A deficient females, whose pregnancy was interrupted for other reasons, failed to show as much as 25% of cornified



causing fetal death and resorption, but also causes developmental anomalies in many of the fetuses that escape intrauterine death.

The widespread interest aroused by the success of Warkany and his collaborators in producing congenital malformations by means of maternal vitamin A deficiency makes it desirable that the work be repeated in another laboratory. To date the only published account of attempts to repeat the experiment elsewhere was that of Jackson and Kinsey,<sup>(5)</sup> who did obtain a few newborn rats with ocular malformations; but the positive results of this investigation were of such limited nature as to cast doubt on the facility and regularity with which such defective young might be produced. One of us (J.G.W.) has been associated with Dr. Warkany in a morphologic and embryologic study of the malformed fetuses and newborns obtained from the experiments carried out in Cincinnati. Since certain material needed for further investigation of this subject (placentae and early embryonic stages prepared especially for histochemical study) was not readily available from Dr. Warkany's laboratory, it was decided to produce it in this laboratory. The present report relates the repetition of the original experiment and, in addition, presents some observations not previously recorded.

*Experimental.* Vitamin A deficiency was induced and maintained by the same diets and experimental procedures as were employed in Warkany's original experiments,<sup>(3,4)</sup> except for the quantity and method of administration of the carotene supplement, and a few other matters of incidental nature specified below. Rats of two strains were used, the Albino Farms and the Wistar; the latter had not been used previously for this type of experiment. Since no difference in reaction to the experimental procedures was detected in animals from the different sources, the matter of strain will receive no further mention. A total of 36 young females were raised from weaning on a semipurified, "preparatory" diet which contained very little vitamin A or carotene. To insure continued growth to maturity

a 15 microgram dose of carotene in olive oil was administered orally each week. Presumably this dosage did not permit appreciable storage of vitamin A. When the animals had attained a body weight of about 160 g, each female was given an opportunity to breed with a male of the same strain fed an adequate diet. If mating occurred, as indicated by presence of sperm in the vaginal smear, the female was transferred to a more highly purified diet which was adequate in all known respects except for the lack of vitamin A. No further supplemental dosing with carotene was given. The day on the morning of which sperm were found in the vaginal smear was considered the first day of pregnancy. Eight control females were fed the same diets and subjected to the same procedures but were given weekly supplements of 150  $\mu$ g of carotene, both prior to and during pregnancy.

From the experience of other investigators,<sup>(1,2,6)</sup> it was expected that a large percentage of the pregnant females on vitamin A deficient diets would resorb all of their conceptuses at some time before term. On the other hand, Warkany and Roth<sup>(6)</sup> found that approximately 25% of the conceptuses which survived until the latter part of pregnancy were developmentally normal. The margin between fetal destruction and normal development, therefore, appears to be very narrow. One of the aims of the present experiment was to attempt to establish criteria by which one might predict ultimate destruction of the conceptuses, thereby making it possible to obtain prior to death embryos which were destined to undergo resorption later. Mason<sup>(1)</sup> observed that the presence of cornified cells in the vaginal smear during pregnancy was frequently associated with subsequent fetal death and resorption. Warkany and Schraffenberger<sup>(4)</sup> considered excessive amounts of blood in the vaginal smear and a loss of weight by the mother as indications that resorption had already begun. It was hoped that by carefully watching for all of these signs (cornified cells in the vaginal smear, excessive blood in the smear, and loss of weight

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6. Warkany, J., and Roth, C. B., *J. Nutrition*, 1948, **35**, 1.

Fig. 2. Abnormal eye of a 22-day rat fetus, from a vitamin A deficient mother. The nervous layer of the retina is deeply folded, contains cysts, and is everted on to the pigmented layer in the region of the optic stalk and along the persistent choroid fissure. The vitreous chamber is reduced to a small cone-shaped volume which is filled with fibrous connective tissue and remnants of the hyaloid artery. Retinal coloboma was present but is not apparent in this section.  $\times 25$ .

Fig. 3. Ectopic openings of ureters in a 19-day rat fetus from an A deficient mother. The left ureter is seen to terminate in the urethra, at some distance below its usual termination at the base of the bladder (normally attained by the 17th day). The right ureter emptied at an even lower level in the urethra.  $\times 25$ .

Fig. 4. Horseshoe kidney in a 19-day rat fetus from an A deficient mother.  $\times 25$ .

Fig. 5. Persisting interventricular foramen in the heart of a 19-day rat fetus from an A deficient mother. The foramen is normally closed by the 17th day of gestation.  $\times 25$ .

Fig. 6. Right-sided aorta and right-sided ductus arteriosus in a 19-day rat fetus from an A deficient mother. The left subclavian artery is also anomalous in that it arises from the descending aorta and passes behind the esophagus to reach the left shoulder.  $\times 25$ .

cells at any time during pregnancy; and both yielded some living young.

Blood in the vaginal smear, in excess of or at times other than that seen during the placental sign, was noted in all of the pregnancies which were later interrupted (Table I). This would seem to be in itself an excellent indicator of fetal destruction, were it not for the fact that a subjective estimate of the quantity of blood is necessary when it appears at the same time as the placental sign. A further limitation of this criterion was noted in that, prior to the 15th day, death and partial resorption of all fetuses often occurred before excessive blood was present in the vagina. On the other hand, when resorption of some conceptuses began during the last third of pregnancy, the quantity of abnormal blood was roughly proportional to the extent of fetal destruction. Two controls showed what was judged to be abnormal blood, and in both cases there was evidence that resorption of some fetuses had begun late in pregnancy.

Loss of weight in the mother was recorded for all but 2 of the pregnancies interrupted prematurely. Also, it was observed in 3 of the controls. The degree of loss was of more significance than the mere fact that a female failed to gain weight. Both the degree and rate of loss appeared to be determined by the number of fetuses undergoing resorption at any one time, as well as by the size of the fetuses at the time of their death.

Thus, of the 3 criteria used in following the course of pregnancy in vitamin A deficient females, no one was found to entirely reliable as an indicator of fetal destruction. However,

when all 3 of these signs became positive during pregnancy, several if not all of the fetuses of that pregnancy were later found to have undergone intra-uterine destruction. Pregnancies interrupted after the appearance of only 2 of the criteria, despite the fact that they were not interrupted immediately, generally yielded some living young.

In an additional group of 14 vitamin A deficient females (not included in Table I) pregnancy was interrupted immediately when the second of any 2 of the signs described above became positive. Accordingly, all of these were killed between the 11th and 15th days. Of this group, 12 females yielded intact embryos, and all but one of these contained more intact than resorbed embryos. This procedure, therefore, proved successful as a means of recognizing impending destruction of the conceptuses, thereby providing embryos and placentae which, although doubtless affected by maternal vitamin A deficiency, had not yet begun disintegration. This material is being prepared for histologic and histo-chemical study of the early stages of abnormality in the embryos and of possible associated changes in the placentae.

*Malformation of the surviving fetuses.* Nineteen of the vitamin A deficient females mentioned in the preceding section yielded offspring suitable for histologic study, but only the fetuses of 15 days gestational age or older have yet been examined for malformations. Of the 7 "litters" comprising the latter group (Table I), all but one contained malformed fetuses. The one litter of normal offspring was from one of the 3 pregnancies which proceeded to term without appreciable indica-

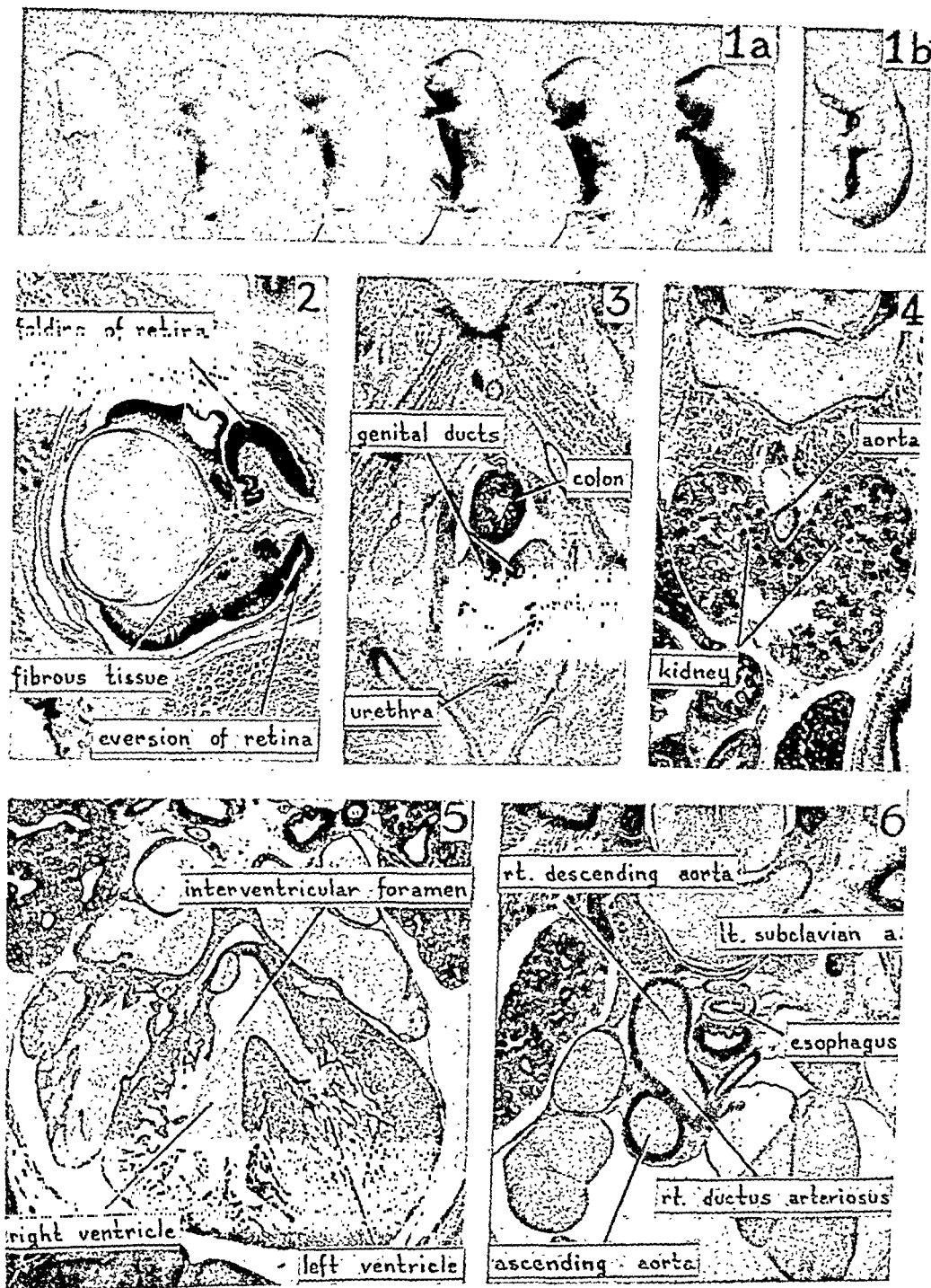


FIG. 1. a. Varying degrees of edema in young rats removed on the 22nd day of gestation from a mother fed vitamin A deficient diets. b. A normal newborn rat from a mother fed vitamin A deficient diets but supplemented with 150  $\mu$ g of carotene per week.

comprising the usual placental sign; and 3) a loss of weight by the mother after the 10th day of pregnancy.

Malformations involving the eye, cardiovascular system, and genito-urinary tract

were found in the fetuses or newborns from all but one of the pregnancies from which intact offspring were obtained after the 14th day of gestation.

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### Beef Erythrocyte Extracts Reacting with Hemagglutinins in Infectious Mononucleosis and in Horse Serum Sickness. (17544)

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In previous publications(1,2) it was demonstrated that 2 serologically active substances can be isolated from the stroma of beef erythrocytes; one reacts with the hemagglutinins that are formed in infectious mononucleosis, the other, with those formed in horse serum sickness. The stroma of beef erythrocytes was first exhaustively extracted with acetone and then with 100% ethanol at room temperature, thus removing serologically inactive substances to a large extent. In order to isolate the specific reactants the stroma residue was then extracted with boiling 100% ethanol, and subsequently, with boiling 80% ethanol. As can be seen from Table I, the 80% ethanol extract yields the so-called mononucleosis antigen (M.A.) and the extract obtained with boiling 100% ethanol, the so-called serum sickness antigen (S.S.A.). Complete and distinct separation of the two serologically active substances could not be accomplished. The fractions do not correspond to pure haptens. However, on the basis of serological activity it could be assumed that the individual fractions were only 2-6% contaminated by the heterogenic fractions. Since the individual fractions are serologically highly active it was thought probable that they might demonstrate significant chemical differences which might

point to the nature of the individual haptens. On the basis of previous investigations the nature of these haptens was disclosed only to the extent that they were found to be thermostable, not digestible by trypsin and pepsin, and did not give reactions characteristic for proteins. This report deals with the chemical analysis of the serologically active fractions of the beef erythrocytes. In addition, the glucose and glucosamine content after hydrolysis is presented.

*Methods.* 1. The C-, H- and N- determinations were done according to Pregl's method of quantitative elementary micro-analysis.

2. Phosphorus was determined as ammoniumphosphomolybdate.

3. The reducing power was determined according to the Hagedorn-Jensen method and calculated for the glucose equivalent. The fractions were hydrolyzed at 100°C with 2 N HCl for 2 hours, then neutralized.

4. The glucosamine determination was done according to the method of Boyer and Fuerth as modified by Brunius.(3) 25-75 mg of dry substance were suspended in 7.5 ml of 4 N HCl and diluted with distilled water to 10 ml. This was hydrolyzed at 100°C in a sealed test tube for 5 hours. After filtration, whenever necessary, color clarification was effected by charcoal adsorption. This solution was carried to dryness over KOH and redissolved in 2 ml of distilled water. This, after

\* This work was carried out by grant in aid from the Roche Research Fund, Basel, Switzerland.

1. Schwarzweiss, H., and Tomcsik, J., *Proc. Soc. Exp. Biol. and Med.*, 1948, v69, 558.

2. Tomcsik, J., and Schwarzweiss, H., *Proc. Soc. Exp. Biol. and Med.*, 1948, v69, 562.

3. Brunius, F. E., *Chemical Studies on the True Forssman Hapten, etc.*, A. B. Fahlerantz Boktryckeri, Stockholm, 1936, p. 141-144.

tions of fetal destruction. No malformations or other abnormalities were found in the offspring of the control animals, which were fed the same diets and subjected to the same procedures as the A deficient mothers except that the controls were given 150 micrograms of carotene per week; both prior to and during pregnancy.

From the 6 abnormal litters older than the 14th day, at least 2 and in most cases all of the young have been serially sectioned and studied. Abnormality of the older fetuses was evident before sectioning by external appearance alone: the characteristic edema of head, neck and thorax was apparent to a variable degree in all (Fig. 1 a and b). Hemorrhage in the region of the eye and prenatal opening of the lids were occasionally noted.

Warkany and collaborators(7) have reported that ocular abnormality was the most prevalent type of developmental defect resulting from material vitamin A deficiency. This was substantiated by microscopic study of animals from the 6 abnormal litters listed in Table I. All such animals examined had ocular anomalies, the more common of which were: folding and eversion of the retina, presence of a mass of fibrous connective tissue in the much reduced vitreous chamber, persistence of the choroidal fissure (coloboma), and absence of the ciliary body (Fig. 2).

Genito-urinary organs also were frequently found to be the sites of malformation in earlier observations.(8) The same types of defects have again been encountered in a similar percentage of animals. Among the commoner abnormalities in both instances were hypoplasia of renal parenchyma, renal ectopia, ectopic ureteric openings (Fig. 3), incomplete development of Müllerian ducts, abnormal retention of heterologous genital ducts in both sexes, aplasia of vagina and faulty differentiation of the urogenital sinus. Less frequent abnormalities were horseshoe kidneys (Fig. 4), cryptorchidism, hypospadias, and atresia of genital ducts and ureters.

Cardiovascular anomalies were found in 44% of a selected group of fetuses and newborns from vitamin A deficient mothers in a previous study.(9) In the present, somewhat smaller group of animals an even higher incidence (more than 60%) of malformations of the heart and major arteries was observed. In general, the anomalies were of the same type, but a few varieties not previously seen were encountered. Failure of the interventricular septum to close was again the most common defect of the heart (Fig. 5). Variations in the aortic-arch pattern frequently observed in both instances were: distally-arising right subclavian artery, right-sided arch of aorta (Fig. 6), double arch of aorta, absence of 4th arch, absence or bilateral persistence of the ductus arteriosus, and absence of a pulmonary artery.

The occurrence of keratinizing metaplasia in epithelia of the lower genito-urinary tract of the offspring from A deficient mothers has been interpreted as indicating that such offspring had themselves experienced *in utero* a state of deficiency similar to that in the mother.(10) The same interpretation may be applied to the present group of animals, in which metaplastic keratinization was again observed in the genito-urinary tract of fetuses older than 18 days gestational age.

**Summary.** This report constitutes a confirmation of the original experiments of Warkany and co-workers in which congenital malformations were induced in fetal and newborn rats by maintaining the mothers on vitamin A deficient diets. In addition, a method has been standardized which permits prior recognition of imminent death and resorption of the conceptuses, thereby making it possible to collect many severely malformed fetuses that would otherwise be destroyed *in utero*. Three criteria that proved reliable in anticipating prenatal destruction of fetuses were: 1) the presence of more than 25% of cornified cells in the vaginal smear at any time during pregnancy; 2) the presence of blood in the vaginal smear in excess of or at times other than that

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## Vitamin D Intake and Susceptibility of Mice to Experimental Swine Influenza Virus Infection.\*† (17545)

GEORGE A. YOUNG, JR., NORMAN R. UNDERDAHL, AND LAWRENCE E. CARPENTER  
(Introduced by Jerome T. Syverton)

From the Hormel Institute, University of Minnesota, Austin, Minn.

Considerable interest has been manifested in recent years in the influence of nutrition on experimental infection. Reviews on the subject have been written by Aycock and Lutman,(1) Schneider,(2) and more recently by Clark *et al.*(3) More specifically related to the results presented in this paper is the work of Sprunt(4) who demonstrated that mice, maintained on low protein diets, showed, after injection with methionine, an increased susceptibility to swine influenza virus infection. Similarly, Midzuno(5) was able to produce influenza in rats by maintaining them on diets deficient in vitamin D. He also carried out similar experiments with avitaminotic D mice. In this paper results are presented which indicate that mice fed diets low in vitamin D are more susceptible to experimental swine influenza virus infection than are mice that receive vitamin D. These findings confirm those reported by Midzuno.(5)

*Materials and methods. Experimental animals.* The mice were obtained by 3 succes-

sive matings of 75 females and 35 males of second generation stock derived from Bittner's ZBC strain(6) with a commercial strain of Swiss mice. The breeding colony was maintained on Purina Fox Checkers and water. To assure uniformity in the ages of the test mice, all breedings for each replicate of the experiment were carried out within a period of 7 days. Mice were weaned at 21 days, and then placed on the experimental diets for 28 days. Thus, at the time of inoculation with swine influenza virus, the mice were at least 49 days old and not more than 56 days old.

*Experimental diets.* The basal diet was prepared by hand mixing all the ingredients listed in Table I, except the brewers' yeast, which was added later as the vehicle for vitamin D. The source of vitamin D was Fleischman's FIDY yeast type 9F containing 9000 U.S.P. units of vitamin D per gram. It was premixed with Fleischman's 2019 dry brewers' yeast. The desired levels of vitamin D were obtained by adding different quantities of the mixture to the diets. For the first experiment, the following amounts of vitamin D were incorporated into the diets: 0, 2.5, 5, 10, 50, 100, 250, 500 and 1000 U.S.P. units per 100 g of feed. In the second replicate experiment, 0, 10, 50, 100 and 500 U.S.P. units per 100 g of feed were used and in the third replicate experiment, 0, 2.5, 5, 10 and 100 units were fed. The diets were fed for 38 days including the 10 days following intranasal instillation of swine influenza virus into 593 test mice. Controls consisted of 123 mice also kept on the different diets for 38 days.

*Mouse infectivity.* The test virus was swine influenza virus (Shope 15) adapted to both eggs and mice. The mice were infected intranasally with 0.05 cc of a virus suspension

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TABLE I.  
Extracts of Beef Stroma and Their Inhibition Titer Toward Sheep Cell Agglutinins.

Extraction	Extracted substance in %	Inhibition titer toward sheep hemagglutinins in	
		Inf. mononuc.	Serum sickness
Acetone, room temp.	8.6	0	0
Ethanol, 100% room temp.	12.6	250	16,000
" 100% boiling	0.7	16,000	250,000
" 80% "	5.1	1,000,000	16,000

TABLE II.  
Chemical Analysis of the Ethanol Extracts of Beef Stroma.

% of	Ethanol, 100% room temp.	Ethanol, 100% boiling	Ethanol, 80% boiling
C	68.13	67.16	56.61
H	11.88	10.54	7.74
N	3.04	3.47	8.45
P	0.35	0.37	2.44
Reducing sugar	2.7	8.31	17.4
Glucosamine	1.76	4.03	8.56

addition of acetylacetone and  $N Na_2CO_3$ , was heated on the water bath and acidified with acetic acid; Ehrlich's reagent was added, air was blown through, and the volume was diluted to 10 ml with glacial acetic acid. The intensity of the red color obtained was determined with a Klett-Summerson photoelectric colorimeter. The calibration curve within the range of 0.2-1.4 mg of glucosamine, was approximately a straight line. The glucosamine content of the unknown solution was interpolated according to this calibration curve.

**Results.** The serological activities of the various beef stroma fractions are shown in Table I. The results of their chemical analyses (average values of several determinations) are given in Table II.

The analytical values of the S.S.A. containing substance, (extracted with boiling 100% ethanol), shows a higher content of reducing sugar and glucosamine than the slightly active fraction obtained with 100% ethanol at room temperature. Significantly different analytical values were found in the

M.A. containing substance which had been extracted with 80% ethanol; here the C- and H- content were lower, the content of N- and P-, as well as that of the reducing sugar and of glucosamine content, were considerably higher. After further purification(1) of the M.A. extract, both its serological activity and reducing properties were somewhat increased (reducing sugar 19.4%). The inactive acetone extract was tested only for reducing substances after the Hagedorn-Jensen method (reducing sugar 0.23%).

The chemical nature of the serologically active M.A. and S.S.A. containing fractions of the beef erythrocytes cannot be determined because of the lack of purity of the preparations. However, it may be inferred that both substances, especially the so-called mononucleosis antigen, belong to the same group of substances as the human A substance and the Forssman antigen, and that glucose or glucosamine play an important role in the hapten structure.

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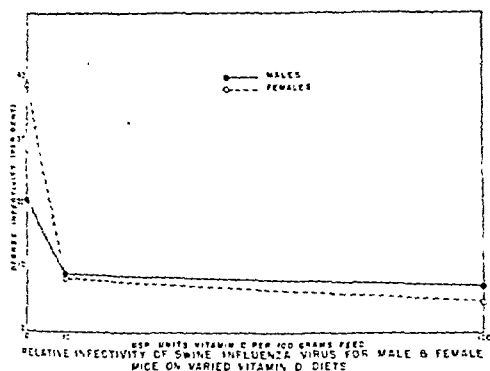


FIG. 2.

graded amounts of vitamin D as the test animals but which were given no virus failed to show evidence of pulmonary consolidation, although the lungs of mice on the lower levels of vitamin D appeared hyperemic. Evidence for vitamin D deficiency was not observed. The mean weight for all mice at the time of weaning was 9.3 g and at the time of infection 22.4 g, the gain in weight having been progressive for all mice to the time of inoculation.

The evidence presented graphically in Fig. 2 shows that susceptibility to infection reflects a sexual difference, as well as a relationship to the dosage of vitamin D. The females were more susceptible than males on diets with no added vitamin D. The differences between males and females on diets containing 10 and 100 U.S.P. units of vitamin D were negligible. Statistically, females on an avitaminotic D diet were significantly more susceptible than males on the same diet, or than females on a diet containing 10 units of vitamin D per 100 g of feed ( $\chi^2 = 12.6$  and  $43.4$ ,  $df = 1$ ,  $P$  less than  $0.1\%$ ). Likewise males on the diet containing no added vitamin D were more susceptible than males on the 10 units diet ( $\chi^2 = 8.6$ ,  $df = 1$ ,  $P$  less than  $1\%$ ).

*Summary.* Experimental evidence is presented which indicates that mice fed diets containing little or no vitamin D for 28 days after weaning showed increased susceptibility to experimental swine influenza virus infection. Females were more susceptible than males.

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## Histochemical Localization of True Lipase.\* (17546)

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In the course of experiments on the histochemical specificity of esterases (lipases) 35 different water-soluble substrates† were tried. Chemically, the substrates were long-chained ( $C_{12}$  to  $C_{18}$ ) fatty acid esters of polyglycols or of sorbitan in which the remaining hydroxyl groups were etherified with ethylene oxide

chains of various lengths. The fatty acids of these compounds were mostly saturated (lauric, palmitic, and stearic); however, a number of them contained unsaturated acids, either practically exclusively (undecylenic, oleic, and ricinoleic) or predominantly (fatty acids of linseed oil).

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† Obtained by courtesy of the Atlas Powder Co., Wilmington, Del., the Onyx Oil and Chemical Co., Jersey City, N.J., the Glyco Products Co., Brooklyn, N. Y., and the General Aniline and Film Corp., New York City.

*Experimental.* Tissues of several species (man, dog, cat, guinea pig, rabbit, rhesus monkey, rat, mouse, and pigeon) were used. They were fixed in chilled acetone and embedded through acetone, alcohol, alcohol-ether, 4% collodion in alcohol-ether (12 hr) and 2 changes of chloroform, 1 hr each, in paraffin. As many as 20 different tissues were included in a single paraffin block. The in-



TABLE I.  
Basal Experimental Diet.

Corn starch			72%
Casein (Borden 52 edible)			18%
Corn oil			4%
Salts			4%
CaHPO <sub>4</sub>	46.3%	FeSO <sub>4</sub>	2.10
CaCO <sub>3</sub>	16.9	KI <sub>4</sub>	.02
NaCl	13.5	ZnO	.04
MgCO <sub>3</sub>	8.5	CuSO <sub>4</sub>	.02
KCl	6.7	MnCl <sub>2</sub>	.02
KH <sub>2</sub> PO <sub>4</sub>	5.7		
Vitamins			Trace
Vit. A	900 U.S.P. units	100 g diet	
Choline chloride	200 mg	"	
Inositol	21.6 mg	"	
Pantothenic acid	4.4 mg	"	
Vit. E	4.0 mg	"	
Niacin	4.0 mg	"	
Paraminobenzoic acid	4.0 mg	"	
Riboflavin	1.6 mg	"	
Thiamine hydrochloride	0.8 mg	"	
Pyridoxine hydrochloride	0.8 mg	"	
Brewers' yeast			2%
			100%

diluted in buffered saline to approximately the minimum infective dose<sub>50</sub>. By the method of Reed and Muench,(7) the dilution for the ID<sub>50</sub> was found to be 10<sup>5.4</sup> for mice on a diet containing 100 U.S.P. units of vitamin D. In the first and third experiments, a dilution of 10<sup>5.7</sup> of allantoic fluid was used, obtained from embryonated eggs harvested 48 hours after inoculation with 10<sup>3</sup> dilution of stock virus. The dilution of virus used for infecting mice was determined by preliminary tests on a small number of mice on a diet containing 100 U.S.P. units of vitamin D. In the second experiment, the preliminary virus titration was misleading, resulting in a 10<sup>7.0</sup> dilution for inoculation of the mice.

The method for determining the extent of mouse infectivity was essentially that of Horsfall(8) and of Lauffer and Miller.(9) Mice dying of influenza within the 10-day test period were each given a score of 4, and those

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that showed consolidation of  $\frac{3}{4}$ ,  $\frac{1}{2}$ ,  $\frac{1}{4}$ , or a trace of the lung area at autopsy on the 10th day were given scores of 3, 2, 1, and 0.5, respectively. The final positive scores differed from those of Lauffer and Miller in that the total score for each test group was divided by four times the number of mice rather than by the total number of mice. This variation made it possible to represent infectivity as a percental number of the maximal possible score that would result from death of all inoculated mice.

**Results.** Fig. 1 shows graphically the relative reactivity of the mice which had been maintained on graded doses of vitamin D to experimental infection with swine influenza virus. This curve, based on the infection of 593 mice, indicates clearly that mice fed for 28 days on a diet containing essentially no vitamin D were more susceptible to experimental infection than mice receiving some vitamin D in their diet. As little as 2.5 U.S.P. units of vitamin D per 100 g of diet afforded definite protection, and 5.0 units gave nearly maximal protection. The differences in relative infectivity of mice on the O diet as compared to those on the 2.5 diet, and the mice on the 2.5 diet as compared to those on the 5.0 diet were significant ( $\chi^2 = 9.36$  and 7.74, df = 1, P less than 1%). There were no significant differences between the other adjacent points on Fig. 1. Points for 250, 500, and 1000 units of vitamin D are not shown on the curve, as they would only extend the curve parallel to the abscissa.

The control mice which were fed the same

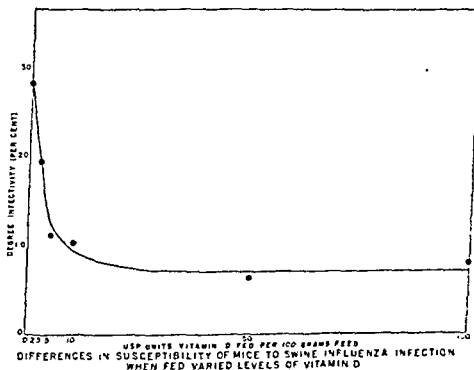


FIG. 1.

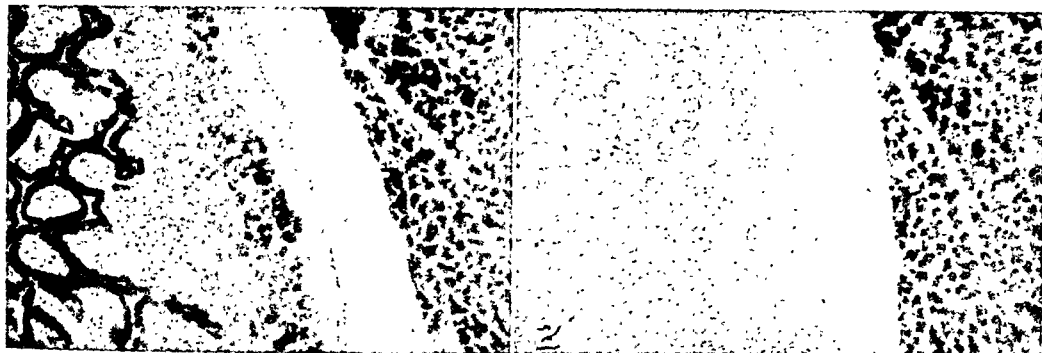


Fig. 1 (left). Pancreas and duodenum of the mouse. Substrate, Tween 60. Intense reaction in both the pancreas and the intestine.

Fig. 2 (right). Next section of the same block. Substrate, Tween 80. Reaction positive in the pancreas only; no reaction in intestine.

*Comment.* The differences between hepatic and pancreatic esterase have long been recognized, the most conspicuous feature of the pancreatic enzyme being its ability to hydrolyze any fatty acid ester, true fats included, while the action of hepatic enzyme is limited to simpler esters. These differences have been analyzed by numerous workers, and a number of important features have been emphasized such as the different nature of their substrates (aliphatic *vs.* aromatic; (6) straight *vs.* branched chains; (6) optical isomerism; (7,8) importance of the chain length of the fatty acid; (6,9) differences in patterns of hydrolysis of short-chained fatty acid esters of nitrophenol), (10) and differences in the effects of activators and inhibitors. (5) The ability of the pancreatic enzyme to attack true fats has led to the usage of designating it as lipase, while the hepatic enzyme is usually referred to as esterase, although both types of enzymes are sometimes included in either of the terms mentioned. To avoid confusion, it is suggested that esterase(s) hydrolyzing true fats (as the pancreatic and gastric enzyme)

be called true lipase(s). The results presented add a new distinctive feature for the characterization of true lipases, namely, their unique ability to hydrolyze esters of unsaturated fatty acids at a fast rate.

Some unsaturated fatty acids are known to be essential in the nutrition of the rat. (11,12) In cystic fibrosis of the pancreas of children it has been shown (13) that the iodine number of serum lipids is lowered, thus indicating the possibility of a selectively poorer utilization of unsaturated fats as compared to that of saturated ones. Whether the low iodine numbers of serum lipids in infantile eczema (14-16) are also due to pancreatic hypofunction is not known. The dependence of some of the symptoms on a deficiency of unsaturated fatty acids is a moot question because it has never been ascertained that the latter are essential in human nutrition. However, should they prove to be so, the results of the experiments presented would indicate

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cubating mixtures were prepared by adding 1 ml of a 5% stock solution of the various substrates to 50 ml of a 0.04 M tris(hydroxymethyl)aminomethane-maleate buffer(1) of pH 7.2 to 7.6, containing 0.2% of  $\text{CaCl}_2$ . A few crystals of camphor were added to prevent bacterial growth. Incubation time was 6 to 18 hr. The precipitates of Ca soaps were visualized by the original method.(2) Chemical controls were run by assaying the enzymatic activity of filtered homogenates of tissues according to a turbidimetric technic(1,3) The substrates were used in a concentration of 1% (somewhat less than 0.01 M, the molecular weights of the substrates being in the range of about 1200 to 1600).

**Results.** All substrates gave consistently good positive reactions with the exception of the undecylenic ester (CRL-16838; Atlas Powder Co.) with which only a few fair results were obtained, besides a large number of partial or complete failures. This may be explained by Ca undecylenate not being sufficiently insoluble. There was a considerable variation in the intensity of the reaction given by the various substrates. On the whole, laurates gave a more intense reaction than palmitates, and the latter a more intense one than stearates. Very long ethylene oxide side chains (as in G-2153; Atlas Powder Co.) seemed to slow down the reaction. However, the most striking differences were observed when results obtained with the use of esters of saturated fatty acids were compared with those given by unsaturated esters. The pictures published previously(4) were obtained exclusively with saturated substrates while with unsaturated substrates the localization of the reaction was greatly restricted, being invariably and intensely positive only in the pancreases of all species (especially the rat and the mouse), and in certain cells in the stomachs of some species (man, monkey, mouse). The liver of the pigeon stained

rather intensely; that of man and of the guinea pig, to a much lesser degree; livers of the other species were practically negative, and so was the intestinal mucosa of most species (man, rat, mouse, rabbit); in others (monkey and pigeon) the intestinal mucosa did show some reaction but it was very much weaker than that obtained with saturated substrates. Whether these faint reactions were due to the presence of small amounts of saturated esters in all substrates or to a slight action of esterases on unsaturated ones could not be decided. They were completely abolished by the addition of 0.005 M taurocholate to the incubating mixture while the reaction in the pancreas was markedly intensified. This selective effect of bile acid on pancreatic lipase has been described previously.(5) Usually there was also some positive reaction in the intestinal contents and on the surface of villi; it appeared to be mostly extracellular and probably due to pancreatic juice or to bacterial enzyme. The kidneys of all species positive with the original technic (mouse, rat, dog, rabbit, monkey) were consistently negative when unsaturated substrates were used. The best substrates to show this restricted reaction were Tween 80, G-2144, G-9446N and G-7627DJ (oleates); G-6486 (ricinoleate) and G-9926T (linseed), all of the Atlas Powder Co.; Neutronyx R (oleate) of the Onyx Oil and Chemical Co., and Antarox B290 (ricinoleate) of the General Aniline and Film Corp. Ricinoleates, as a rule, gave distinctly weaker but even more selective reactions than either the oleates or the linseed ester. It should be remarked that the unsaturated substrates do not seem to diffuse through collodion membranes; therefore, if they are to be used, the slides must not be collodionized: in fact, the celloidin from the embedding procedure should be removed with alcohol-ether.

Chemical assays were in complete agreement with the histochemical findings. Table I shows the activity of hepatic and pancreatic extracts towards a number of substrates; it is obvious that while the pancreatic enzyme attacks all of them, the hepatic enzyme leaves the unsaturated ones practically untouched.

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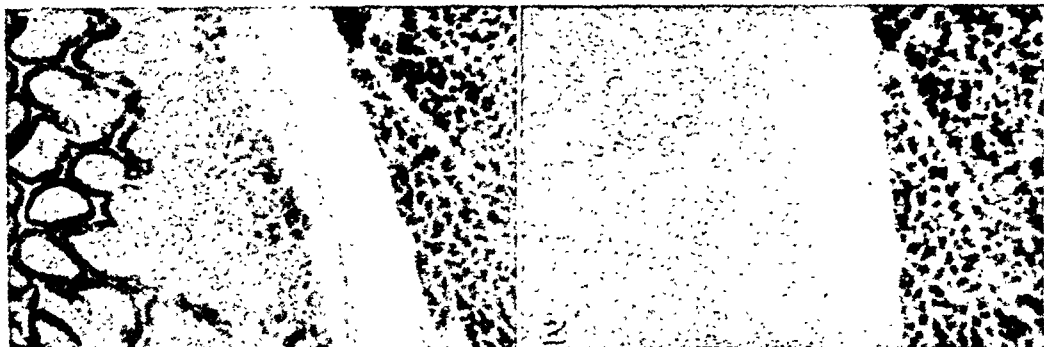


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incubating mixtures were prepared by adding 1 ml of a 5% stock solution of the various substrates to 50 ml of a 0.04 M tris(hydroxymethyl)aminomethane-maleate buffer(1) of pH 7.2 to 7.6, containing 0.2% of  $\text{CaCl}_2$ . A few crystals of camphor were added to prevent bacterial growth. Incubation time was 6 to 18 hr. The precipitates of Ca soaps were visualized by the original method.(2) Chemical controls were run by assaying the enzymatic activity of filtered homogenates of tissues according to a turbidimetric technic(1,3) The substrates were used in a concentration of 1% (somewhat less than 0.01 M, the molecular weights of the substrates being in the range of about 1200 to 1600).

**Results.** All substrates gave consistently good positive reactions with the exception of the undecylenic ester (CRL-16838; Atlas Powder Co.) with which only a few fair results were obtained, besides a large number of partial or complete failures. This may be explained by Ca undecylenate not being sufficiently insoluble. There was a considerable variation in the intensity of the reaction given by the various substrates. On the whole, laurates gave a more intense reaction than palmitates, and the latter a more intense one than stearates. Very long ethylene oxide side chains (as in G-2153; Atlas Powder Co.) seemed to slow down the reaction. However, the most striking differences were observed when results obtained with the use of esters of saturated fatty acids were compared with those given by unsaturated esters. The pictures published previously(4) were obtained exclusively with saturated substrates while with unsaturated substrates the localization of the reaction was greatly restricted, being invariably and intensely positive only in the pancreases of all species (especially the rat and the mouse), and in certain cells in the stomachs of some species (man, monkey, mouse). The liver of the pigeon stained

rather intensely; that of man and of the guinea pig, to a much lesser degree; livers of the other species were practically negative, and so was the intestinal mucosa of most species (man, rat, mouse, rabbit); in others (monkey and pigeon) the intestinal mucosa did show some reaction but it was very much weaker than that obtained with saturated substrates. Whether these faint reactions were due to the presence of small amounts of saturated esters in all substrates or to a slight action of esterases on unsaturated ones could not be decided. They were completely abolished by the addition of 0.005 M taurocholate to the incubating mixture while the reaction in the pancreas was markedly intensified. This selective effect of bile acid on pancreatic lipase has been described previously.(5) Usually there was also some positive reaction in the intestinal contents and on the surface of villi; it appeared to be mostly extracellular and probably due to pancreatic juice or to bacterial enzyme. The kidneys of all species positive with the original technic (mouse, rat, dog, rabbit, monkey) were consistently negative when unsaturated substrates were used. The best substrates to show this restricted reaction were Tween 80, G-2144, G-9446N and G-7627DJ (oleates); G-6486 (ricinoleate) and G-9926T (linseed), all of the Atlas Powder Co.; Neutronyx R (oleate) of the Onyx Oil and Chemical Co., and Antarox B290 (ricinoleate) of the General Aniline and Film Corp. Ricinoleates, as a rule, gave distinctly weaker but even more selective reactions than either the oleates or the linseed ester. It should be remarked that the unsaturated substrates do not seem to diffuse through collodion membranes; therefore, if they are to be used, the slides must not be collodionized: in fact, the celloidin from the embedding procedure should be removed with alcohol-ether.

Chemical assays were in complete agreement with the histochemical findings. Table I shows the activity of hepatic and pancreatic extracts towards a number of substrates; it is obvious that while the pancreatic enzyme attacks all of them, the hepatic enzyme leaves the unsaturated ones practically untouched.

1. Gomori, G., *PROC. SOC. EXP. BIOL. AND MED.*, 1948, v68, 354.

2. Gomori, G., *PROC. SOC. EXP. BIOL. AND MED.*, 1945, v58, 362.

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4. Gomori, G., *Arch. Path.*, 1946, v41, 121.

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TABLE I.  
Conversion of Desoxycorticosterone to Glycogenic Material.

Exp.	Amt of desoxy- corticosterone equivalent added, mg	Adrenal tissue added, wet wt, g	No. of mice used	Equivalent of 11-dehydro- corticosterone found, mg	Net amt of glycogenic material formed calculated as 11-dehydro- corticosterone, mg	Conversion, %
	0	5 (Slices)	6	< .20	—	—
	0	5 "	5	.20	—	—
	0	5 (Homogenate)	5	< .20	—	—
A	100 (Free)	4.5 (Slices)				
		1.5 (Homogenate)	7	3.84	3.60	3.6
B	67.0 (Glucoside)*	4.5 (Slices)	7	4.40	4.16	6.2
		1.5 (Homogenate)	10	1.92	1.72	5.1
C	33.5 "	5 (Slices)	10	3.89	3.71	11.1
D	33.5 "	3.5 (Homogenate)	10	< .3		
	33.5 "	—	10			

\* This preparation was a 1% solution of the glucoside containing 10% glucose and 10% acetamide.

ing. The combined acetone filtrates were evaporated to an aqueous sludge, 25 ml of distilled water added, and brought to pH 7.0. This aqueous residue was extracted 3 times with ethylene dichloride. The ethylene dichloride extract was concentrated to dryness and taken up in corn oil for bioassay by a mouse glycogen method.(2) The results are expressed in 11-dehydrocorticosterone equivalent.

The results are summarized in Table I. Five grams of adrenal tissue incubated without steroid yielded 0.2 mg equivalent of 11-dehydrocorticosterone or less. For the calculation of net amount of glycogenic material formed the value of 0.04 mg of 11-dehydrocorticosterone per g of tissue was used as the amount of activity material contributed by the tissue as such. The unreacted desoxycorticosterone contributed a negligible amount of glycogenic activity.

Incubation of desoxycorticosterone either with slices or homogenate resulted in the formation of glycogenic material. The yields were higher when the glucoside was used. Incubation of the glucoside with the homogenate gave a higher yield of glycogenic material than when slices were employed (Table I).

Since glycogenic activity in the adrenalectomized mouse is associated with steroids having oxygen at carbon 11, it is presumed that the enzyme system is concerned with the oxidation of carbon 11 to either a carbonyl or an alcohol grouping.

**Summary.** Incubation of desoxycorticosterone either as the free compound or as the glucoside with adrenal slices or homogenate resulted in the formation of glycogenic material.

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TABLE I.

Hydrolysis of Water-soluble Fatty Acid Esters by Hepatic and Pancreatic Esterase.  
Buffered substrate, 10 ml; 4% tissue extract (rat liver and pancreas), 0.5 ml. Activities expressed in micromoles of fatty acid liberated in 1 hr.

	Tween 20 (lauric)	Tween 40 (palmitic)	Tween 60 (stearic)	CRL-16838 (undecylenic)	G-2144 (oleic)	G-6486T (ricinoleic)	G-9926T (linseed)
Liver	6.6	3.8	3.3	0.4	0.4	0.0	0.5
Pancreas	40.0	19.6	10.4	16.8	16.6	3.0	19.2

that in attempts at therapy they must be administered pre-hydrolyzed, as soaps and not as fats, since hydrolysis of the latter in the intestinal tract is likely to be inadequate. It should be remarked that in the human intestine even esterase reaction is weak.

*Summary.* Water-soluble unsaturated fatty acid esters are hydrolyzed almost exclusively by the pancreatic type of esterase (true

lipase) while similar esters of saturated fatty acids are readily attacked by esterases of both the hepatic and pancreatic type. This difference is utilized in a technic for the histochemical localization of true lipase activity. The findings presented may have implications in human pathology.

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### Metabolism of the Steroid Hormones. Conversion of Desoxycorticosterone to Glycogenic Material *In vitro*.<sup>\*</sup> (17547)

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Recently, Hechter *et al.* (1) have reported the conversion of desoxycorticosterone to corticosterone by the isolated adrenal gland using a perfusion technic. We have been able to demonstrate the conversion of desoxycorticosterone into material possessing glycogenic properties with adrenal slices and an adrenal homogenate preparation.

Desoxycorticosterone,<sup>†</sup> either as the free compound or the glucoside, was incubated for 6 hours at 38°C with adrenal slices, homo-

genates, or a combination of both. In Experiments A, B, C (Table I) where adrenal slices were used, 50 mg of steroid were dissolved or suspended in a total volume of 25 ml containing 0.01 M glucose, 0.062 M sodium chloride, and 0.02 M sodium phosphate buffer (pH 7.4). The gas phase was air. In Experiment D, 50 mg of steroid was contained in a total of 25 ml of media containing 5.0 ml of a 10% boiled adrenal cortical extract in saline, 0.01 M sodium fumarate, 0.062 M sodium chloride, 0.025 M potassium chloride, and 0.004 M magnesium sulfate. The gas phase was air.

At the end of incubation, 400 ml of acetone were added and the mixture placed in the cold overnight. The precipitated tissue was filtered with suction, ground, and extracted 3 times with hot acetone. Tissue slices were cut into small pieces with a scissors before grind-

<sup>\*</sup> Supported in part by a grant (RG 1050) from the Division of Grants and Fellowships, National Institutes of Health, U. S. Public Health Service.

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<sup>†</sup> The desoxycorticosterone and desoxycorticosterone glucoside were generously supplied by Ciba Pharmaceutical Products, Inc.

# Bacterial Activators (*Lysokinases*) of the Fibrinolytic Enzyme System of Serum.\* (17549)

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In recent years, revived interest in a proteolytic enzyme system of the blood has resulted in new data and some difficulties as to terminology.(1,2)

In view of the practical importance of fibrinolytic phenomena, we have accepted the "fibrinolysin" rather than the "trypsin" or "plasmin" nomenclatures. A brief summary of these terminologies and our abbreviations follows: 1. *Lysin* or fibrinolysin (trypsin, plasmin): the active proteolytic (fibrinolytic) enzyme; 2. *Antilysin* or antifibrinolysin (antitrypsin, antiplasmin): inhibitor of lysin; 3. *Prolysin* or profibrinolysin (tryptogen, plasminogen): the inactive precursor of lysin; 4. *Lysokinase* (tryptokinase): any activator of prolysin, e.g., streptokinase, (3) staphylokinase, (4) fibrinokinase; (5) 5. *Antilyso-kinase* (antitryptokinase): inhibitor(s) of lysokinase(s), e.g., antistreptokinase.(6)

The present study concerns methods and results of an extensive survey of available bacterial types for *lysokinase* activity. Earlier studies(3,7) showed that streptokinase specifically activates only human prolysin, while staphylokinase affects prolysin from human, dog, guinea pig, and rabbit sera but not other species tested.(8) This further search for

new bacterial lysokinases and particularly for an activator of bovine prolysin has been somewhat discouraging.

**Materials.** *Thrombin.* Upjohn's bovine thrombin (courtesy Dr. J. T. Correll), 20 units per cc aqueous (sterile) solution. *Fibrinogen:* 1% aqueous solution of Armour's bovine "Fibrinogen," pH =  $7.0 \pm 0.2$ , sterilized by Seitz filtration. *Prolysin preparations:* A. For Survey Tests—(1) Human Plasma Fraction-I<sup>†</sup> (courtesy Dr. J. T. Edsall), 2% aqueous (sterile) solution, containing fibrinogen as well as prolysin; (2) Dog and Bovine Sera, sterilized by Seitz filtration: B. For Lysokinase Tests—(3) Human, Dog, and Bovine I Serum Fractions, prepared from pooled sera, at 0°C, by precipitation at 25% concentration of ethanol, the deposit after one hour being recovered by centrifugation and redissolved in one-half the original serum volume of borate buffer(7) (pH = 7.75) and stored frozen at -20°C; (4) Bovine II, a fraction prepared by 33% saturation of serum with  $(\text{NH}_4)_2\text{SO}_4$ , at 1°C, as previously described.(7)

**Bacterial cultures.** Stock cultures from the Bacteriology Laboratory of the University of North Carolina were obtained through the courtesy of Drs. D. A. MacPherson and F. L. Rights. The  $\beta$ -hemolytic streptococcus (No. 98) control culture was obtained from Dr. Maxwell Finland of the Thorndike Memorial Laboratory (Boston). Additional cultures isolated from bovine sources were made available through the courtesy of Dr. L. T. Giltner, U. S. Dept. of Agriculture, and Dr. H. E.

S. Gerheim, E. B., and Ferguson, J. H., *Proc. Soc. Exp. Biol. and Med.*, 1949, v71, 261.

† This product was developed from blood, collected by the American Red Cross, by the Department of Physical Chemistry, Harvard Medical School, under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

\* This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

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2. Ferguson, J. H., *Quarterly of Phi Beta Pi*, 1948, v44, 279.

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7. Lewis, J. H., and Ferguson, J. H., *J. Clin. Invest.*, in press.



# Influence of Desoxycorticosterone on Cesium Excretion in Adrenalectomized Rats.\* (17548)

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Adrenal cortical steroids are known to influence the metabolism of sodium and potassium, both members of Group I of the periodic table. We have studied the possible influence of one of the adrenal cortical hormones, desoxycorticosterone, on the excretion of administered cesium, the heaviest member of Group I.

The method employed has been described previously for radio-sodium(1) and for radio-potassium(2) and need only be mentioned briefly here. Male rats purchased from Holtzman (Madison, Wis.) were bilaterally adrenalectomized. Twenty-four hours after operation the animals were injected subcutaneously with 2 different doses of desoxycorticosterone† dissolved in 0.25 cc of corn oil. Control rats received only 0.25 cc of oil. One hour after receiving the hormone the rats were injected with 2 cc of an aqueous Cs<sub>134</sub>‡ solution containing 10.3 µg of cesium carbonate. The rats were immediately placed in glass metabolism cages and the urine collected directly into

ointment tins for a period of 6 hours. The urines were evaporated to dryness and radio-cesium determined as previously described for radiosodium.(1) The cesium excretion for the experimental period was expressed as the percentage of the material administered.

Table I lists the results which indicate an excretion of  $5.20 \pm 0.75\%$  of the administered cesium within the 6 hour period. The administration of 25 and 250 µg of desoxycorticosterone had no significant influence on the cesium excretion. As little as one microgram of the hormone caused a significant retention of sodium(1) and 10 µg caused a significantly increased excretion of potassium.(2)

**Summary.** Desoxycorticosterone did not influence the excretion of administered radio-cesium in adrenalectomized rats. Under specific conditions of the test  $5.20\% \pm 0.75\%$  of the administered radiocesium was excreted in the urine within the first 6 hours.

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TABLE I.  
Influence of Desoxycorticosterone on Cesium Excretion in Adrenalectomized Rats.  
14 rats in each experiment.

Desoxycorticosterone, µg	Mean body wt, g $\pm$ S.E.	Mean excretion of Cs <sup>134</sup> , % $\pm$ S.E.	Significance of difference. “t”
0	144 $\pm$ 2	5.20 $\pm$ .75	—
25	138 $\pm$ 2	4.28 $\pm$ .45	1.03
250	138 $\pm$ 3	4.87 $\pm$ .73	0.31

\* Supported in part by a research grant (RG 234) from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service, and a grant from Sharp and Dohme, Inc.

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2. Dorfman, R. I., *Proc. Soc. Exp. Biol. and Med.*, 1949, v70, 732.

† The desoxycorticosterone was kindly supplied by Ciba Pharmaceutical Products, Inc.

‡ The radiocesium was obtained from Monsanto Chemical Co. through the U. S. Atomic Energy Commission.

TABLE I.  
Survey of Fibrinolytic (I) and Lysokinase (II-IV) Effects of Certain Bacteria (See text).

Bacterial type	I Control	II Human	III Dog	IV Bovine	Comment
* <i>Streptococcus pyogenes</i> ( $\beta$ -hemolytic)	0	++++	0	0	No. 98 (Finland)
* <i>Staphylococcus aureus</i> (hemolytic) ( <i>Micrococcus pyogenes</i> , var. <i>aureus</i> )	0	++++	++++	0	Gelatine liq.
* <i>Staphylococcus aureus</i> † (hemolytic)	0	++++	++++	0	" "
" "	0	++	++++	0	" "
" "	0	++	++++	0	" "
<i>Streptococcus pyogenes</i>	0	++++	0	0	
" <i>mitis</i> ( <i>viridans</i> )	0	+++	0	0	
* <i>Sarcina lutea</i>	0	++++	+	0	" "
* <i>Pseudomonas aeruginosa</i>	+	+	++++	+	" "
* <i>Klebsiella pneumoniae</i> ( <i>friedlanderii</i> )	++	++	+++	++	
" "	0	0	++	0	
* <i>Serratia marcescens</i>	++	++++	0	0	" "
* <i>Salmonella paratyphi</i>	0	+++	++	0	
" "	0	0	+	0	
" "	++	++	++	0	
" <i>schottmuelleri</i>	++	0	+	0	
" <i>typhimurium</i> ( <i>aertrycke</i> )	+++	+	+	+	
" "	++	0	++	0	
" <i>choleraesuis</i>	+++	+	+++	0	
" "	++	++	0	0	
" <i>enteritidis</i> ( <i>gaertneri</i> )	+	+++	0	0	
" <i>anatidis</i>	+++	0	++	0	
* <i>Shigella ambigua</i>	0	+++	0	0	
" "	+	+	+	0	
<i>Brucella (abortus) suis</i> (in O <sub>2</sub> )	++++	0	0	0	
" " (in CO <sub>2</sub> )	++++	0	0	0	
* <i>Bacillus subtilis</i>	+	0	+++	+	" "
" "	+	0	+++	0	" "
" "	++++	++	++++	+	" "
<i>Clostridium septicum</i>	++++	++++	++++	+++	" "
" <i>botulinum</i>	++++	++++	++++	+++	" "
" "	++++	++++	++++	+	" "
" <i>perfringens</i> ( <i>welchii</i> )	++++	++++	+++	+++	" "

\* Cultures subsequently selected for lysokinase tests.

† Bacteria isolated from bovine sources.

subsequent lysokinase studies (see below). The streptococcus and staphylococcus strains listed separately at the top of Table I are typical good lysokinase producers and have been used repeatedly in this laboratory for preparation of streptokinase and staphylokinase, respectively. Strains isolated from bovine sources are indicated (†).

**Lysokinase study.** As indicated in Table I, lysokinase studies (see *Methods*) were attempted on alcohol-precipitated material isolated from 12 bacterial strains, namely, 1.  $\beta$ -hemolytic *Strep. pyogenes* (human source), 2. hemolytic *Staph. aureus* (human source), 3. hemolytic *Staph. aureus* (bovine source), 4. *Sarcina lutea*, 5. *Pseudomonas aeruginosa*, 6. *Klebsiella pneumoniae* (Friedlander), 7.

8. *Serratia marcescens*, 9. *Salmonella paratyphi*, 10. *Salm. typhimurium* (Aertrycke), 11. *Shig. ambigua*, 12. *Bacillus subtilis*, 13. *Clostridium botulinum*.

Fibrinolytic tests (7) were performed after preliminary incubation of "lysokinase" preparations with the 4 prolysins (see *Materials*: B. (3), (4)) as follows: (1) no incubation (tested immediately), (2) 20 min. at 37°C., (3) 1 hour at 29°C., (4) 24 hours at 6°C. The staphylokinase of bovine origin (hemolytic *Staph. aureus*) activated both human and dog prolysins to approximately the same lytic strength as our standard (human) staphylokinase. It did not affect bovine prolysin, however. The material from *Clostridium botulinum* (anaerobic) cultures was

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**Stock medium for survey tests.** "Yeast peptone," containing yeast extract (1%), tryptose (2%), dextrose (0.2%), NaCl (1.6%), pH =  $7.6 \pm 0.2$ , sterilized by autoclaving. This *double strength* stock medium was diluted by the other materials of the test mixtures (see below). Uninoculated mixtures of medium plus fibrin clots, with and without added prolysin (human, dog, bovine), showed no fibrinolysis.

**Methods.** The first procedure was a general survey of all available bacterial cultures for fibrinolytic activity as manifested during growth in medium + fibrin clots, with and without added prolysin. Secondly, selected cultures showing positive results in the survey test, were followed up with attempted isolation of a partially purified lysokinase by the standard alcoholic precipitation of culture filtrates as used for streptokinase(9) and staphylokinase.(4)

**Survey method.** For aerobes, sterile fibrin clots (with nutrient medium), in  $13 \times 100$  mm tubes, were inoculated from 24 hour broth subcultures by stabbing and seeding of surface. For anaerobes, the clots were doubled in volume and covered with paraffin. Control (no prolysin) and prolysin-containing clots were incubated at  $37^\circ\text{C}$  and observed after 24 and 48 hours. Bacterial tubes in which growth was not satisfactory were repeated or discarded and in those reported growth was successful. Each bacterial strain was tested in the following 4 clot mixtures: I. Control (no prolysin): 1 cc medium + 1 cc fibrinogen + 0.2 cc thrombin; II. Human: 1 cc medium + 1.5 cc Human Plasma Fraction-I (prolysin and fibrinogen) + 0.2 cc thrombin; III. Dog: 0.5 cc medium + 0.5 cc dog serum (prolysin) + 1.0 cc fibrinogen + 0.2 cc thrombin; IV. Bovine: 0.5 cc medium + 0.5 cc bovine serum (prolysin) + 1.0 cc fibrinogen + 0.2 cc thrombin.

**Lysokinase study.** Bacteria selected as a result of the survey test (see above) were cultured for 24 hours in 500 cc Bacto heart infusion broth and centrifuged subsequently to

remove suspended bacteria. The selected anaerobe (*Cl. botulinus*) was grown in thio-glycollate broth. The "lysokinase," in each instance, was prepared by the alcoholic precipitation method of Garner and Tillett,(9) yielding a dry powder, subsequently prepared for use as a 1% solution in borate buffer and tested for activation of human, dog, and bovine prolysin (see *Materials*), utilizing methods previously described.(7)

**Results. Survey study.** Seventy-seven cultures of 40 types of bacteria were satisfactorily submitted to the lysis tests of the 4 types noted under *Methods*. Some common organisms were omitted because cultures were unavailable at the time or, in some cases, because of failure to grow under the test conditions. Completely negative results (no significant lysis of any of the clots) were obtained with: *Streptococcus pyogenes* (5 strains, incl. 1 erysipelatis—but cf. Table I), *Strep. faecalis*, *Strep. acidominum* (uberis), *Strep. mitis* (viridans—3 strains—but cf. Table I); *Micrococcus* (Staphylococcus) *citreus*, *Micr. pyogenes*, var. *albus* (*Staph. albus*—2 strains); *Gaffleya tetragena*; *Neisseria catarrhalis*; *Corynebacterium pseudodiphtheriticum*; *Vibrio metschnikovii*; *Alcaligenes faecalis*; *Escherichia coli* (3 strains); *Aerobacter aerogenes* (3 strains); *Proteus vulgaris* (1 strain); *Salmonella* (*Eberthella*) *typhosa* (2 strains); *Shigella paradysenteriae* (7 strains). *Shig. sonnei* (2 strains); *Pasturella multocida* (*suilla*), *Past. pseudotuberculosis*; *Brucella melitensis*,<sup>‡</sup> *Bruc. abortus*,<sup>‡</sup> *Bacillus megatherium*; *Mycobacterium phlei*.

Table I summarizes the survey tests on all bacteria which produced significant fibrinolysis in any of the clots, whether in the control (I), without prolysin, or in the presence of human (II), dog (III), or bovine (IV) prolysin. The degree of lysis is roughly quantitated 0—++++. Under *Comment* is noted the ability to liquefy gelatine, and the lack of correlation between this property and the fibrinolytic phenomena is noteworthy. Individual cultures of the same type often varied in the results of these tests. The strains asterisked in Table I were selected for the

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<sup>‡</sup> Grown both aerobically and in  $\text{CO}_2$ .

TABLE I.  
Effect of Fertile Eggs Upon Deterioration of Aureomycin at 37°C.

Hr of incubation	Medium		
	Infusion broth	Intact egg	Homogenized egg
0	50.*	50	50
24	<12.5	25-50	25-50
48	2.0	32	16
72	—	16	8
96	2.0	—	6
144	1.0	12	4
168	0.5	12	3

\* Aureomycin concentrations ( $\mu\text{g/ml}$ ).

signed to test such a possibility, it was found that when 7-day old embryonated eggs were inoculated by the yolk sac route with aureomycin most of the aureomycin activity could still be demonstrated in the egg fluids as long as 7 days after inoculation. The presence of actively multiplying psittacine virus did not affect the persistence of the aureomycin activity. The following experiments were, therefore, performed to determine whether a substance exists in eggs which permits aureomycin to retain its potency despite prolonged incubation under conditions which usually destroy the activity of the antibiotic agent.

**Methods.** Aureomycin hydrochloride<sup>‡</sup> was made up to the desired concentration in brain heart infusion broth (Difco), pH 7.2. Aureomycin levels were determined by a serial 2-fold dilution method similar to that used for penicillin (10) using separate pipettes for each dilution. *Bacillus* No. 5<sup>§</sup> was the standard test organism. All test fluids were incubated at 37°C.

**Experimental. Tests in Embryonated Eggs.** In the first experiments it was sought to determine whether the protective factor was found only in relation to intact chick embryos. Seven-day old embryonated eggs, with an average weight of 61.0 g and an assumed average volume of 40.0 ml of fluid were used. A dose of 2.0 mg of aureomycin in 0.25 ml of broth was injected into the allantoic sac of each embryo. Control eggs received the same amount of broth containing no drug.

<sup>‡</sup> Supplied in crystalline form in sterile vials by Lederle Laboratories.

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<sup>§</sup> Obtained from Dr. B. M. Duggar.

Three uninoculated embryonated eggs were each homogenized aseptically in a Waring blender; 2.0 mg of aureomycin in broth were added to 2 of them and a comparable volume of broth alone was added to the third. The homogenized eggs and the intact fertile eggs were then incubated. At intervals thereafter fertile eggs were homogenized and their contents of aureomycin assayed and compared with those in the eggs originally homogenized. A control tube containing 2.0 mg of aureomycin in 40 ml of broth was also included. The results appear in Table I. It is seen that both in the intact and in the homogenized embryos the rate of deterioration of aureomycin at 37° was much slower than in the broth. The protective effect was probably unrelated to differences in hydrogen ion concentration:—the pH of recently homogenized eggs, as determined with a Beckman potentiometer, was found to vary between 7.1 and 7.3; that of homogenized eggs after prolonged incubation, was 7.1-7.2, and that of the control broth was 7.2. In no case did eggs without aureomycin exert any significant effect on the growth of the test organism.

**Tests with Nonfertile Eggs.** Nonfertile eggs were examined for their capacity to delay the deterioration of aureomycin, and the relative potencies of yolk and albumen, in this regard, were also determined. An egg showing no evidence of fertility was homogenized in the blender and 1.0 mg of aureomycin added to 20 ml of the emulsion. The yolk and albumen of another nonfertile egg were separated aseptically and aureomycin added to each in the same concentration. Broth and a 25% solution of human serum albumin, each containing the same concentration of aureomycin,

actively fibrinolytic but lysed clots containing prolynsins in longer times than the prolysin-free control (lysis time: 15 min.) so that its action is presumably due solely to a proteolytic enzyme from the bacteria themselves. All other materials gave negative results in concentrations of 0.06-1.0%. There were insufficient materials for testing at higher concentrations.

**Discussion.** Omitted from these studies were a number of bacterial types, some of which were known to have been tested by earlier workers with the older "fibrinolytic" technics. Tillett(10) reviews a number of these and particularly states that the pneumococcus gives negative results. Our data clearly show that fibrinolytic activity is encountered in cultures of a wide variety of bacteria, but only in the case of certain hemolytic streptococci and staphylococci can a definite lysokinase be demonstrated. The results with

*Clostridium botulinum* show the need for the prolysin-free control test. Fibrinolytic activity appears to be unrelated to the gelatine liquefaction test.

**Summary.** In a survey test of 77 satisfactory cultures of 40 bacterial types, fibrinolytic phenomena were encountered in 19 types (33 cultures) of bacteria tested. Only certain hemolytic streptococci and staphylococci gave positive results with a specific test for *lysokinase*, however, and these organisms were not directly fibrinolytic nor able to activate bovine profibrinolysin. *Direct* fibrinolytic activity may occur in cultures when (except in the case of *Cl. botulinum*) it is not found in the alcoholic precipitate. The failure to obtain a lysokinase in at least 6 types of bacterial cultures which gave good results in the survey test is disappointing, as is the failure to find a kinase for bovine profibrinolysin.

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## A Substance in Egg Yolk Which Inhibits Deterioration of Aureomycin Activity.\* (17550)

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In the treatment of chick embryos infected with psittacosis virus(1) the single dose of aureomycin required to protect 50% of infected embryos for 7-9 days was found to be one-third to one-fourth as great as the dose of chloromycetin required to produce the same effect. This finding was unexpected in view of the rapidity with which aureomycin deteriorates *in vitro* at 37°C under neutral or slightly alkaline conditions(2-6) as well as the relative stability of chloramphenicol under similar

circumstances.(7-9) It appeared possible, therefore, that some factor, present in the embryonated egg, protected aureomycin from deterioration. In preliminary experiments, de-

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## Fractionation of Amino Acid Mixtures in Acetone by Means of Alkyl Acid Phosphates.\* (17551)

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In a former communication(1) we described the property of solvating amino acids in dry acetone or dioxane, which is possessed by 8 organic acids, di- and trichloroacetic acids, and certain sulfonic acids. The purpose of the present communication is to state that we have found that several alkyl acid phosphates† also possess the property of solvating a dry mixture of amino acids in dry acetone. These may be used for dividing a protein hydrolysate into a series of fractions containing one to six or seven amino acids, certain of these composing a high percentage of the individual fractions. On adding ammonia to acetone solutions of amino acids formed by the aid of alkyl acid phosphates, both amino acids and the ammonium salts of the reagent-acids precipitate together. This property of alkyl acid phosphates contrasts strongly with that of many sulfonic acids, which, under these conditions, form salts with ammonia which are readily soluble in acetone, permitting the precipitated amino acids to be separated from the solvating agent. The triethylamine (T.E.A.) salts of the alkyl acid phosphates are soluble in acetone. This property permits the amino acids to be separated from the reagent by means of this base.

Individual amino acids show marked differences in solubility in an acetone solution of octyl, n-amyl, iso-amyl, n-butyl, iso-butyl, n-propyl and iso-propyl acid phosphates. Table I illustrates the contrasting solvating power of 5 reagent acids for 9 amino acids. We designate by the term proline equivalent, the ratio of the amount of a reagent necessary to dissolve one equivalent of amino acid to the

amount of the same reagent necessary to dissolve one equivalent of proline.

The amino acids in a hydrolysate exert marked influence on each other's solubility in acetone-alkyl acid phosphate solutions. Much less acetone-reagent is required to dissolve a given weight of dry protein hydrolysate than is necessary to dissolve the component amino acids of the sample in the isolated state. There is, however, pronounced selectivity in the order in which certain amino acids are dissolved when successive portions of an acetone-reagent are passed through a sample of hydrolysate contained in a filtering crucible.

Table II illustrates some contrasting properties of alkyl acid phosphates in solvating power for a casein hydrolysate.‡ It also shows the differences in extent of precipitation of amino acids from their acetone-reagent-hydrolysate systems after neutralization with triethylamine, and the degree of complexity of the more difficultly soluble residues not brought into solution in our experiments.

*A Procedure for Fractionation of a Protein Hydrolysate.* A 10 g sample of the dry, finely ground, hydrolysate is thoroughly mixed with an equal weight of anhydrous calcium sulfate (Drierite), and the mixture is placed in a sintered glass crucible equipped with filter flask and suction connection. A test tube is placed inside the filter flask to collect the filtrate. The amino acid-Drierite mixture is thoroughly wet with the reagent (e.g. a 0.2 N solution of an alkyl acid phosphate in acetone), the contents of the crucible are stirred for a definite time (usually one minute), suction is applied, and the solution in the crucible is sucked through into the test tube. Another portion of the reagent is then added, and the

\* This investigation was supported in part by a grant-in-aid from Nutrition Foundation, Inc.

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† The authors are indebted to Monsanto Chemical Co., St. Louis, Mo., through the courtesy of Mr. Paul Logue, for the samples of alkyl acid phosphates employed in this investigation.

‡ The authors are indebted to National Dairy Products Corp. Research Laboratories, Oakdale, L.I., through the courtesy of Dr. Arnold H. Johnson, for the sample of hydrochloric acid casein hydrolysate, their "Hycase," a salt-free preparation.

TABLE II.  
Effect of Nonfertile Eggs, Egg Yolk and Egg Albumen on Deterioration of Aureomycin at 37°C.

Hr of incubation	Medium				25% human serum albumin
	Infusion broth	Homogenized nonfertile egg	Egg yolk	Egg albumen	
0	64.0*	64	64	64	64.0
24	8.0	32	64	8	16.0
72	2.0	16	32	4	0.5
144	1.0	4	16	4	<0.1

\* Aureomycin concentrations ( $\mu\text{g/ml}$ ).

served as controls. Aureomycin levels were determined immediately and at intervals after incubation. The results, which appear in Table II, show that the nonfertile egg also has the capacity to protect aureomycin from deterioration. Furthermore, the protective substance was found in the yolk. Thus, whereas the broth controls and the tubes containing egg and serum albumins retained but one-eighth of the original activity after 24 hours of incubation, there was no significant loss in the presence of whole egg or egg yolk alone. Even after 72 hours of incubation, when a 32-fold loss had occurred in the broth controls and a 16-fold loss in the presence of egg albumen, there was but a 4-fold deterioration in the presence of whole egg and a 2-fold deterioration in the presence of egg yolk. A 2- to 4-fold variation in activity has generally been considered to be within the limits of error of the method. The effect of dilution of egg yolk on its capacity to protect aureomycin from loss of potency is demonstrated in Table III. Egg yolks from nonfertile eggs were mixed with broth in varying proportions and sufficient aureomycin added to achieve a standard concentration of 50  $\mu\text{g}$  per ml. It is seen that when egg yolk has been diluted beyond 1:4 its capacity to protect aureomycin from deterioration diminishes.

Subsequent experiments have shown that the protective substance in yolk resists heating at 60°C for 80 minutes. Studies still in progress indicate that this protective substance remains active after heating for one hour at 100°C and, under certain conditions, even after autoclaving at 120° for 15 minutes.

*Discussion.* The rapid rate of deterioration of aureomycin activity upon standing has been a constant source of concern to those

TABLE III.  
Inhibition of Deterioration of Aureomycin at 37°C by Varying Dilutions of Egg Yolk in Broth.

Final dilution of egg yolk	Hr of incubation		
	0	24	72
1:2	32*	32	8
1:4	32	32	4
1:10	32	16	2
1:20	32	16	2
1:100	32	8	1
Broth alone	32	8	1

\* Aureomycin concentrations ( $\mu\text{g/ml}$ ).

working with this antibiotic. Stringent precautions are necessary to avoid loss of aureomycin activity in body fluids withdrawn for assay, and even then aureomycin deteriorates during the period of incubation required for assay.(3) Whether similar deterioration occurs within the body is not clear; however, significantly large amounts of injected aureomycin remain unaccounted for after assay of body fluids. The mechanism of the destruction of aureomycin is quite unknown,(6) but it is manifestly of value to seek a substance which will inhibit this destruction. It has been shown in the present studies that such a substance exists in egg yolk. Further studies on the isolation, characterization and properties of this substance are now in progress.

*Summary.* A substance (or substances) found in egg yolk, inhibits the deterioration of aureomycin activity which ordinarily occurs upon incubation at 37°C. The protective agent is heat stable and of sufficient concentration to permit dilution of egg yolk to 1:4 without significant diminution of aureomycin-protective action. Egg albumen and human serum albumin are not protective in the concentrations used.

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**Summary.** Seven alkyl acid phosphates have been found able to solvate amino acids in dry acetone. Marked differences in solvating power are shown by these reagents for individual amino acids, and for selective solvation of amino acids from complex mixtures such as a protein hydrolysate. Mixtures of amino acids greatly influence each others' solubility when fractionation is effected by the method described.

When systems consisting of acetone-amino acid-solvating reagent, are neutralized with

triethylamine, amino acids are precipitated, but never quantitatively. Marked differences occur in the extent to which the dissolved amino acids precipitate with this treatment; e.g. 90% were precipitated from the n-propyl, as against but 45% from the isopropyl system, when the amino acids were solvated from a dry casein hydrolysate.

These reagents offer prospects for usefulness in the fractionation of amino acid mixtures.

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## The Cardiac Output and Circulation Time of Ferrets.\* (17552)

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The ferret was first used as a laboratory animal by Dunkin and Laidlaw for the study of canine distemper.(1) Subsequently it was also found to be susceptible, under experimental conditions, to other viral infections such as influenza,(2) fowlpox,(3) swineherd's disease,(4) Rift Valley fever,(5) a strain of poliomyelitis virus,(6) and lymphocytic chorio-meningitis.(7) In influenzal infection, experimental investigations employing ferrets have contributed a great deal to our knowledge of the pathology(8) and epidemiology(9) of this important disease. Other

aspects of the infection, such as the problem of the toxicity of the virus and the alterations in the infected host's physiological functions are fields relatively unexplored. It seems that the ferret, because of its larger size, might serve better than the mouse or other rodents for investigations of these problems. Except for a general description by Pyle,(10) we are not aware of any detailed report dealing with the normal physiology of this animal. The present study dealing with the cardio-vascular function of normal ferrets was initiated in order to obtain such data before investigations of pathological physiology were undertaken. Cardiac output determined by the direct Fick principle, arterial blood pressure, and circulation time measurements were selected to depict the status of the animal's cardio-vascular functions.

**Materials and methods.** 1. *Animals:* North American ferrets (*Putorius putorius*)‡ of both sexes with body weights varying from 700 to 1100 g were used. The body surface was calculated by the formula: (11) Surface area in square meters = (body weight in g)<sup>2/3</sup> ×

\* This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service.

† Research fellow in Medicine, University of Illinois College of Medicine.

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‡ Identified by Mr. K. P. Schmidt, curator of zoology, Natural History Museum, Chicago.



TABLE I.  
 Proline Equivalents of Alkyl Acid Phosphates in 0.2 N Solutions in Acetone.

Amino acid	Octyl	n-Amyl	n-Butyl	n-Propyl	Isopropyl
Proline	1	1	1	1	1
Isoleucine	3	2	4	3	1
Ph. alanine	6	6	7	2	2
Leucine	6	4	5	13	2
HO-proline	12	7	14	5	3
Tryptophan	14	3	7	2	1
Threonine	16	5	7	4	2
Serine	42	42	19	9	11
Glutamic acid	58	70	42	23	8

 TABLE II.  
 Solvating Power of 0.2 N Solutions of Alkyl Acid Phosphates, Precipitates Yielded on Neutralization with Triethylamine (T.E.A.), and Chromatographs of an Undissolved Residue.

Alkyl acid phosphate	% of sample of a casein hydrolysate readily soluble†	% of soluble material precipitated on addition of T.E.A.	Results of chromatographing the insoluble material	Remarks
Octyl	97	85	—	
n-Amyl	45	80	—	*
Iso-amyl	95	75	3 spots	
n-Butyl	97	68	—	
Iso-butyl	97	80	1 spot	Tyrosine
n-Propyl	97	90	2 spots	
Iso-propyl	100	45	—	†

\* The amyl acid phosphate-insoluble material has been provisionally identified as the basic amino acids, methionine, tyrosine (and possibly serine), alanine, proline, valine, and the leucines. This insoluble fraction contains all of the tyrosine. The moiety which is soluble in amyl acid phosphate, and precipitable with triethylamine, has been tentatively identified as a mixture of some of each of the basic amino acids, aspartic acid, glutamic acid (and possibly serine), phenylalanine, and/or valine, alanine, methionine, proline, and the leucines. All of the glutamic and aspartic acids are found in the soluble and precipitable material. The soluble material which remains in solution after neutralization with T.E.A. appears to contain almost all of the proline.

† The portion of the hydrolysate which precipitates from solution with iso-propyl acid phosphate after the reagent is neutralized with T.E.A. exhibits 6 spots when examined by ascending chromatography using 77% ethanol. These spots have been shown to be arginine, histidine, lysine, glutamic acid (and possibly serine), aspartic acid, alanine, and the leucines. Glutamic acid, lysine and arginine predominate in this mixture. Histidine and aspartic acid are present only in traces.

process of stirring and filtration by suction is repeated until the desired volume of filtrate has been collected. The apparatus is then disassembled, the test tube containing the filtrate is replaced by an empty one, the apparatus reassembled, and the process of fractionation continued. The size of the filtrate fractions taken off will be determined by the number of fractions it is desired to make from the hydrolysate sample. We have made as many as 50 to 60 fractions in this manner, but experience has shown that, for our objectives, 10 to 20 fractions are all that it is worth while to make in the first fractionation. The fractions thus secured are precipitated by neutralization of the solvating acid reagent by addition of triethylamine, whereupon a certain

portion of the dissolved amino acids is precipitated, as shown in Table II.

*Fractionation with Amyl Acid Phosphate.*  
 When a sample of casein hydrolysate is fractionated with 0.2 N amyl acid phosphate in acetone according to the procedure described, the resulting fractions, when chromatographed, show that much simplification has occurred as compared with the original hydrolysate. The first increments of reagent which are added dissolve greater weights of hydrolysate per cubic millimeter than do later additions. These facts indicate that the solvating action of the reagent is a selective one, and that the undissolved fraction of the hydrolysate is continuously changing in composition as the fractionation proceeds.

TABLE I. Measurements of Circulatory Function of Normal Ferrets.

No.	Sex	Body wt., g	Rectal temp., °F	Respiratory rate, per min.	Pulse rate, per min.	O <sub>2</sub> consumption, cc/min.	O <sub>2</sub> content arterial, %	O <sub>2</sub> content venous, %	A-V O <sub>2</sub> difference, vol., %	Cardiac output, cc per min.	Body surface (m <sup>2</sup> )	Cardiac index (liter/min./m <sup>2</sup> )	Blood pressure, mm Hg	Fluorescein circulation time, sec.	Cyanide-circulation, sec.	Hematocrit, %
1	M	730	101	27	94	7.2	16.3	8.1	8	87.8	.029	1.69	120			49
2	M	800	101.4	13	87	6.0	15.8	10.6	5.2	115.4	.035	2.09	156			35
3	M	850	101.2	14	84	7.5	20.4	12.8	7.6	98.7	.037	1.75	138			46
4	M	780	100.8	26	168	5.5	17.9	11.2	6.7	82.0	.034	1.51	134	6.0	3.9	41
5	M	1120	99.8	26	290	10.4	15.4	9.5	5.9	176.2	.067	2.15	185	8.1	3.1	35
6	M	800	99.3	12	168	3.8	18.7	15.5	3.2	118.7	.055	2.62	175	6.5	5.5	46
7	M	800	100.8	22	278	10.8	13.5	8.1	5.4	200.0	.055	3.62	133	4.5	4.9	41
8	M	700	97.3	38	172	3.9	12.4	6.9	5.5	156.0	.051	3.06	150	8.5	5.2	32
9	M	850	97.0	58	250	9.6	13.2	8.1	5.1	154.9	.055	2.81	150	5.8		41
10	M	830	99.4	58	227	7.2	15.7	8.3	4.9	195.9	.057	3.42	136	6.8		33
Mean		823	99.4	28.6	227	7.2	15.7	10.2	5.5	138.5		2.467	147	1.2	4.5	40
Stand. dev.		114	1.5	13.1	50.5	2.5	2.6	1.8	1.7			0.753	20		0.7	4.7

mm of mercury.

Circulation time measured by the fluorescein technic (6.8 sec.) was longer than that determined by the cyanide method (4.5 sec.), because of the greater distance the dye had to traverse when it was injected through the femoral instead of the superficial neck vein. The cyanide technic gave a sharper endpoint, although it has the objection of including the reaction of the respiratory center in a function test primarily for the circulatory system. The short circulatory time of ferrets rendered it very likely that technical errors such as the rate of injection and promptness in perceiving the endpoint would jeopardize the reliability of the results.

**Discussion.** In this group of animals, all the common conditions known to alter cardiac output as ingestion of food, hemorrhage, exercise and oxygen content of the inspired air(14) had been eliminated or made as uniform as possible. The only uncontrollable factor was the individual variations in response to the effect of the anesthetic used. In dogs, Shore(15) had shown that the effect of barbituric anesthesia on cardiac output was quite variable during the course of their experiments. The differences between successive determinations on the same animals were as high as 35%. Furthermore, anesthesia might cause hypoventilation or atelectasis, both of which would interfere with normal cardiac function. The individual variations in the cardiac output of our normal animals were partly technical and partly real. Our results agreed with those of Warren *et al.* on human subjects(16) in that arterial blood oxygen showed less variable results, provided that hematocrit readings were fairly constant. Greater degrees of variations, however, were encountered in the measurements of oxygen consumption. Because of these variations, they concluded that, "results are valid only when groups rather than individuals are studied."

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1/1000. The validity of the calculated value was carefully checked by actual measurements on the animals.

2. *Circulation time.* The experiments were performed in the morning on fasting animals. Anesthesia was induced by intraperitoneal injection of a solution of sodium nembutal, 30 mg per kg of body weight. Ten minutes later, the animal was placed on a board in supine position. Circulation time was then determined by the use of two technics: (a) A short incision was made in the left inguinal region and the femoral vein isolated. Five-tenths ml of a 10% sodium fluorescein solution was quickly injected into the vein. The interval between the beginning of the injection and the appearance of a green fluorescence in the oral mucous membrane, observed under ultra-violet light, was accurately timed; (b) A superficial vein in the neck was isolated and two-tenths ml of a 1% sodium cyanide solution was injected. The time from the beginning of the injection and the appearance of a deep inspiratory movement was noted.

3. *Cardiac output.* An incision was next made in the midline of the neck exposing the trachea for cannulation. The left common carotid artery was freed and cannulated with a No. 20 needle with a blunt bevel, connected to a mercury manometer which registered on a kymographic drum. The external jugular vein on the opposite side was dissected free and its cephalid end was tied. With the use of a specially constructed forceps(12) having sharp thin tips, an opening was made and held open in the wall of the vein. A No. 025 polythene catheter with metal stylet was introduced into the vein between the opened forceps. The catheter was advanced carefully toward the heart. The final position of the tip of the catheter was ascertained by placing the animal under a fluoroscope and with further manipulations the tip was made to lie in the right ventricle. The stylet was then removed and 0.2 ml of a 10% heparin solution in saline was introduced into the

catheter to prevent clotting. The position of the catheter was fixed by tying a ligature around the vein.

The cardiac output was determined according to the direct Fick principle.<sup>§</sup> The oxygen consumption was measured by connecting the tracheal cannula to a spirometer containing 100% oxygen. The volume of oxygen consumed was readily calculated from the kymographic tracing. As soon as the tracheal cannula was linked to the spirometer, a 1.5 ml sample of arterial blood was withdrawn from a three-way stopcock which was connected to the common carotid artery for the blood pressure determinations. A sample of venous blood (1.5 ml) was then quickly drawn from the polythene catheter. Both samples of blood were drawn into paraffined syringes and delivered immediately under a layer of paraffin oil in tubes. Determinations of the oxygen contents of these samples were made without delay according to the method of Van Slyke and Neil.<sup>(13)</sup> Five-tenths ml samples of blood were used for each determination and duplicates checked within 0.1 vol. %. Before the animal was killed, 1 ml of venous blood was taken from the polythene catheter, heparinized, and hematocrit readings were made by Wintrobe's method.

*Results.* Our findings (Table I) on the average rectal temperature and respiratory rate among the 10 normal animals were lower than those reported by Pyle<sup>(10)</sup> chiefly because our readings were made on anesthetized animals. Ferrets were easily excitable animals and such readings taken on unanesthetized animals were subject to wide fluctuations. The cardiac output, expressed in the form of cardiac index, was calculated in terms of liters per square meter of body surface per minute and was on the average  $2.476 \pm 0.75$  liters. The average oxygen content of arterial blood was 15.7 vol. % and that of venous blood 10.2 vol. %. The average mean arterial blood pressure was 147

§ Cardiac output =

Oxygen consumption in ml per minute

(Arterial O content) — (Venous O content)

13. Peters and Van Slyke, *Quantitative Clinical Chemistry*, Vol. 2, p. 321.

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TABLE I.

Effect of Methyl Folic Acid on Deposition of Alkaline Phosphatase after Treatment with Estradiol.

No. of chicks	Diet	Estradiol, $\mu$ g	Body wt, g	Oviduct wt, mg	Alkaline phosphatase
3	Control	—	95	17.3	0
2	"	50	112	29.0	3+
3	Antagonist	—	70	13.2	0
3	"	50	68	16.2	1+

TABLE II.

Effect of Crude Methyl Folic Acid and a Folic Acid Free Diet on Deposition of Alkaline Phosphatase after Treatment with Stilbestrol.

No. of chicks	Diet	Stilbestrol, mg	Body wt, g	Oviduct wt, mg	Alkaline phosphatase
6	Control	—	157	17.3	3.7+
7	"	0.5	159	436.3	3.7+
5	Synthetic	—	79	12.8	1.6+
7	"	0.5	71	78.5	1.7+
6	Antagonist	—	73	12.2	1.9+
7	"	0.5	68	69.2	0.7+

diet(6) supplemented with all the known vitamins except folic acid. Starting on the 13th day of feeding the special diets, 7 chicks in each of the 3 diet groups received 0.5 mg stilbestrol daily for 5 days. All chicks were killed 24 hours after the last injection and autopsied as in Exp. 1. In order to obtain information with regard to the relative amount of phosphatase present under the various conditions of the experiments an arbitrary scale was established ranging from 0 reaction for tissue totally devoid of phosphatase to a 4+ reaction for tissue showing a maximum response. In both experiments the same scale was used and the evaluation made only on the phosphatase noted in the endometrium.

**Results.** The chicks on the control diet showed a normal increase in body weight throughout the experiments whereas the birds on the synthetic diet and the diet containing the folic acid antagonist showed negligible increase in weight after the 9th day. Thus the final body weight of the chicks in Exp. 1 on antagonist diet was 69 g while the controls showed a body weight of 101 g. Similarly the control chicks in Experiment 2 reached a final weight of 158 g while the weight of the folic acid deficient chicks varied from 68 to

79 g (Tables I and II). Hemoglobin values varied widely; however, an average drop of 10% was obtained in the folic acid deficient birds. In Exp. 1, treatment with estradiol caused a 68% increase in the oviduct weight of the normal bird but had no effect on the weight of the oviduct of the chicks receiving the folic acid antagonist. Phosphatase occurred to a marked degree in the control chicks which showed a 3+ reaction and only to a slight degree, *i.e.*, a 1+ reaction, in the birds receiving the antagonist (Table I).

In Exp. 2 the higher dosage of estrogen used gave a much greater effect on the weight of the oviduct. The oviducts of the control chicks treated with stilbestrol weighed 436.3 mg whereas the oviducts of the chicks on the synthetic and antagonist diets weighed 78.5 and 69.2 mg respectively (Table II). The alkaline phosphatase was also markedly reduced in the oviducts of these last two groups. It is apparent from an examination of Fig. 1 that a large amount of alkaline phosphatase is present in the chicks on the normal diet whereas the folic acid deficient chicks show a marked reduction in the enzyme regardless of stilbestrol treatment.

**Discussion.** The present study confirms the work of Hertz(7) and others that folic acid is necessary for the growth-promoting

In a table compiled by Marshall(17) the cardiac indices for various animals were: Dog, 2.86; goat, 3.07; rabbit, 1.69; and horse, 5.84. The mean cardiac index of 2.476 for the present group of normal ferrets is one intermediate between those for dog and rabbit.

*Summary.* The mean cardiac output per

minute per square meter of body surface for a group of 10 normal ferrets was found to be 2.476 liters. The average circulation time by the fluorescein technic was 6.8 seconds and 4.5 seconds as determined by the cyanide method. The average mean arterial blood pressure was 147 mm Hg. Various other related data were also tabulated.

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Received November 15, 1949. P.S.E.B.M., 1949, v72.

### Amount of Alkaline Phosphatase in the Oviduct of Folic Acid Deficient Chicks.\* (17553)

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Increases in alkaline phosphatase in the reproductive tract following injection of estrogens have been described for many species. Hertz and Sebrell(1) first noted an impairment in the response to stilbestrol of the oviduct of chicks deficient in folic acid. This failure of estrogens to induce growth in the absence of folic acid has also been observed in the monkey,(2) frog,(3) and rat.(4) The following experiments were undertaken to determine whether the amount of alkaline phosphatase in the oviduct of the chick is affected by a folic acid deficiency.

*Material and methods.* Exp. 1. Eleven white leghorn chicks were obtained at hatching and placed in cages having elevated wire mesh floors. At 2 days of age 5 chicks were placed on a diet of chick mash, while the remainder were fed chick mash containing 2% folic acid

antagonist.† Both groups received water *ad libitum*. Three birds in each group were kept as uninjected controls. The remaining chicks were injected subcutaneously with 50 $\mu$ g of estradiol§ in 0.05 ml of sesame oil daily for 5 days. The injections were started on the 12th day of feeding and the chicks were killed 24 hours after the last injection. At post mortem the oviducts were removed, weighed and placed in cold 80% ethyl alcohol. Histochemical studies for alkaline phosphatase were carried out by the method of Gomori,(5) in which the sections were incubated at 37°C for 2 hours with glycerophosphate and examined without the use of a counterstain. Body weights were recorded daily and blood samples obtained at autopsy for hemoglobin determinations.

*Exp. 2.* A total of 38 white leghorn chicks were obtained at time of hatching and treatment started at 2 days of age. Thirteen of the chicks received the normal diet, 13 received the normal diet containing 2% folic acid antagonist and 12 received a synthetic

\* Aided in part by a grant from the American Cancer Society, Inc., to Harvard University.

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‡ We are indebted to Dr. E. L. R. Stokstad, Lederle Laboratories, New York, for the crude  $\alpha$ -methyl folic acid used in these studies.

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# Effects of X-Rays and Nitrogen Mustard on Lymphatic Nodule of the Rabbit.\* (17554)

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The relationships between the dose and the histologic effects of total body radiation with X-rays on the lymphatic nodule has been reported previously.(1) It was found that the sequence of histologic changes in the lymphatic nodules is characteristic for different dose levels. Total body radiation of rabbits with 800r and 600r at a rate of 16r per minute produces a rapid and complete destruction of the lymphatic nodule resulting in a nodule-free period beginning at 5 days with a subsequent regeneration at 21 days after radiation. Lower doses (400r and lower) produce only a partial destruction of the lymphatic nodule with an immediate regeneration so that no nodule-free period occurs. For a detailed description of the changes in the lymphatic nodule after X-rays, we refer to previous papers.(1) The similarity between the hematotoxic effect of X-rays with that of nitrogen mustard is well known and has been reported by a number of investigators. It seemed to us of interest to investigate whether this similarity extends itself to the histologic effects on the lymphatic nodule and if so, to compare, on this basis, the degree of the effect on the lymphopoietic system of these two agents with respect to their general toxicity to the organism. The nitrogen mustard compound used for this investigation is methyl bis (B-chloroethyl) amine hydrochloride.

The LD<sub>50</sub> dose is taken as a measure of the general toxicity of X-rays and nitrogen mustard. These are respectively 800r (LD<sub>50</sub>/30 days)(2) and 2 mg per kg (LD<sub>50</sub>/14

days).(3) The 0.1% solution of nitrogen mustard was injected immediately after the crystals were completely dissolved in view of the instability of the compound. Eighteen rabbits were injected intravenously with 2 mg per kg nitrogen mustard. Eleven rabbits were killed at the following intervals: 30 min.; 3 hr; 8 hr; 16 hr; 24 hr; 3 days, 5 days, 10 days, 21 days, 6 weeks and 2 months. After the first 4 animals were killed, 7 of the remaining 14 died within 14 days after the injection. This indicates that the given dose was in the range of the LD<sub>50</sub> for this group of animals. The lymphatic nodules of the mesenteric lymph node and the appendix were studied.

The sequence of events in the lymphatic nodule of the mesenteric lymph node of the rabbit after nitrogen mustard is essentially identical with that after irradiation with the LD<sub>50</sub> dose. The destruction of lymphocytes leads to a nodule-free period beginning at 5 days and which terminates at 21 days when regenerative processes are present as evidenced by the formation of new nodules. Comparing the degree of regeneration with that after 800r of X-rays at the same interval, it appears that the regeneration is more advanced after nitrogen mustard. At 21 days there are larger and more new lymphatic nodules. These lymphatic nodules are at this time still without corona of small lymphocytes ("bare" germinal centers). A corona is present at 6 weeks and the regeneration is complete from then on.

In contrast to the changes in the mesenteric lymph node, the changes in the appendix after the LD<sub>50</sub> dose of nitrogen mustard are not comparable with those after an LD<sub>50</sub> dose of X-rays but they are similar to the results after the lower dose levels (400r and less). There is no nodule-free period, but after the debris has cleared up, small groups of lymphocytes remain in the dense masses of reticular

\* This work was supported by a grant from the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

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FIG. 1.

Sections through oviducts, all  $\times 32$ . Note that the heavy deposition of alkaline phosphatase present in the endometrium of A and B, fails to occur in folic acid deficiency (C-F).

A—Uninj. control.

B—Normal diet. Inj. with stilbestrol.

C—Synthetic diet.

D—Synthetic diet. Inj. with stilbestrol.

E—Antagonist diet.

F—Antagonist diet. Inj. with stilbestrol.

action of estrogen on the reproductive tract. The mechanism whereby this vitamin acts in the growth of the tissue is as yet unexplained.

7. Hertz, R., *Recent Progress in Hormone Research*, II, 1948, Academic Press.

It has been suggested that folic acid may function as a coenzyme in the synthesis of thymine-like compounds which are then utilized in the production of nucleic acid.(8) Partial support for this concept is seen in the work of Prusoff *et al.*(9) who showed that a decrease in desoxyribonucleic acid occurred when *Lactobacillus casei* is grown on a folic acid deficient medium. It has also been shown that aminopterin not only inhibited the estrogen induced growth of the oviduct of the frog but caused an increase in the number of mitotic figures, conceivably by a reduction in nucleic acid and consequent retardation in cell division.(10) The present results indicate that folic acid may also be essential for the deposition and maintenance of alkaline phosphatase in the reproductive tract. If this enzyme is necessary for the growth-promoting action of estrogen then its absence would explain the failure of estrogens to induce growth. Thus it is possible that the absence of folic acid as induced under the conditions of this experiment interferes with the increase in phosphatase following estrogen treatment and prevents normal growth response.

**Summary.** A folic acid deficiency produced by feeding a synthetic diet or the addition of crude x-methyl folic acid to a normal diet markedly decreases the amount of alkaline phosphatase in the oviduct of chicks after estrogen treatment.

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9. Prusoff, W. H., Tepley, L. J., and King, C. G., *J. Biol. Chem.*, 1948, v176, 1309.

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TABLE I.  
Blood Chemical Determinations on 4 Cortisone-treated Rabbits and 4 Control Rabbits Following Production of Ear Wounds.

	5th post-operative day				8th post-operative day			
	Cortisone-treated rabbits		Control rabbits		Cortisone-treated rabbits		Control rabbits	
	No. 55	No. 57	No. 52	No. 56	No. 54	No. 58	No. 53	No. 59
Serum protein (g%)	5.1	5.0	—	—	5.7	5.1	5.5	6.0
Serum albumin (g%)	4.2	4.3	—	—	3.8	4.1	3.8	4.1
Serum sugar (mg%)	235	170	235	122	294	129 (fasting)	174	122 (fasting)
Plasma vit. C (mg%)	—	.42	—	.65	—	0.34 (fasting)	—	.02 (fasting)

effects are achieved has not, up to the present, been clarified, but it has appeared to the writers that the action of these agents is on "host reactivity."<sup>(5)</sup> In the course of treatment of several patients with lupus erythematosus disseminatus and rheumatoid arthritis with ACTH, it was noted that open wounds, such as incised abscesses and decubitus ulcers, failed to show normal granulation tissue.<sup>(5)</sup> One such wound, an incised abscess, did not form granulation tissue until 4 days after ACTH was discontinued. Healing of aseptic wounds, made for biopsy, was greatly delayed while ACTH was administered, but these healed promptly when the hormone was discontinued. These observations raised the question of whether the action of ACTH and cortisone in the "mesenchymal diseases" might be due to "inhibition of reactivity" of the connective tissue. Patients with hyperadrenalism as manifested by Cushing's syndrome are known to have a disturbance of connective tissues as exemplified by the "moon" facies, formation of striae, osteoporosis, increased fragility of blood vessels and poor healing of wounds. To test this hypothesis it was decided to determine whether the administration of cortisone might inhibit the normal processes of granulation in artificially produced wounds.

Skin wounds were made on both ears of six pairs of rabbits weighing between 2.4 kg and 3.0 kg.<sup>(6)</sup> One rabbit of each pair was

treated with cortisone according to the following schedule: 12.5 mg of cortisone acetate was administered intramuscularly twice daily for 3 days before the wound was made and for 5 to 8 days thereafter until the time of sacrifice. The other rabbit of each pair served as a control and received no cortisone but was otherwise treated exactly the same. The animals were kept in a thermostatically controlled room and identical local treatment to prevent wound infection, as reported elsewhere,<sup>(6)</sup> was carried out in each pair. Control and cortisone-treated rabbits ate a similar diet, both groups ate well and weight remained relatively constant.

The development of granulation tissue in the cortisone-treated animals was markedly delayed in all cases. Grossly, at both 5 and 8 days following operation, these rabbits showed little or no granulation tissue and the blood vessels of the wound stood out clearly, while the controls showed good granulation tissue which obscured the blood vessels. Two pairs of rabbits were sacrificed on the fifth post-operative day and two pairs were sacrificed on the eighth post-operative day. The remaining two pairs were not sacrificed but showed similar gross changes and are being used for further long-term experiments. The cortisone-treated rabbits developed such manifestations of hyperadrenalism as the disappearance of circulating eosinophils, a moderate postprandial hyperglycemia and a great increase

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6. Howes, E. L., *Surg., Gyn. and Obs.*, 1943, v76, 738.



cells. Regeneration occurs from these remaining lymphocytes.

These observations indicate that the histologic effect of nitrogen mustard on the lymphopoietic system is qualitatively but not quantitatively the same as the effect of X-rays at the same level of general toxicity. The effects on the mesenteric lymph node differ only slightly, the difference being a somewhat earlier regeneration. The difference is greater in the lymphatic tissue of the appendix. This lymphatic tissue shows changes after nitrogen mustard at the LD<sub>50</sub> comparable to the changes of the X-rays with a dose of half the LD<sub>50</sub>. The findings of Jacobson *et al.*(4) demonstrating the more rapid regeneration of the rabbit bone marrow after nitrogen mustard as compared to X-rays and the findings reported here, seem to warrant the conclusion that the hematopoietic system regenerates in general more rapidly after nitrogen mustard than after X-rays with dose levels of the

same general toxicity, keeping in mind local differences such as are reported here for the mesenteric lymph node and the lymphatic tissue of the appendix. We have no definite explanation for these local differences. They may be based on differences in sensitivities of the lymphocytes, but it may also be that the instability of the compound *in vivo* causes differences in the effective concentrations in different organs.

*Summary.* The histologic effects on the lymphatic nodules of the rabbit after X-rays and after nitrogen mustard are identical in nature. However, there is a quantitative difference in the response of the lymphatic nodules to the LD<sub>50</sub> dose of the two agents. The difference is minor in the mesenteric lymph node, where there is a slightly earlier regeneration after nitrogen mustard than after X-rays. The difference is greater in the appendix where the histologic changes after nitrogen mustard at the LD<sub>50</sub> dose level are comparable to the changes after half the LD<sub>50</sub> dose of X-rays.

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### Effect of Cortisone on Production of Granulation Tissue in the Rabbit.\* (17555)

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Since Hench and his co-workers(1) demonstrated the dramatic therapeutic effect of cortisone and adrenocorticotrophic hormone (ACTH) in rheumatoid arthritis, numerous

investigators have found that these agents exert beneficial effects in a great array of disorders. These include such "mesenchymal diseases" as rheumatic fever, lupus erythematosus disseminatus and periarteritis nodosa. (2-4). The mechanism by which therapeutic

\* Supported in part by the Masonic Foundation for Medical Research and Human Welfare, the U. S. P. H. S., and a grant from the Medical Research and Development Board, Office of the Surgeon General, U. S. Army.

<sup>†</sup> Postdoctorate Research Fellow, National Institutes of Health, U. S. Public Health Service.

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FIG. 2.

Cross-section of rabbit's ear wound, 124 $\times$ , eighth post-operative day, cortisone-treated rabbit.

Pre-formed blood vessels left in the connective tissue at the time the skin was removed may be seen in the center of the field. Note the narrow area of granulation tissue with densely-packed fibroblasts.

months before any interference with wound healing is noted. Thus if this effect be related to vitamin C deficiency the result might be due to impairment of utilization of available ascorbic acid. No manifestations of the hemorrhagic diathesis were observed in the animals receiving cortisone. This correlates well with clinical findings in humans since no manifestations of scurvy have developed in patients we have treated with ACTH for as long as 4 months, and 600-900 mg of ascorbic acid administered daily to patients being treated for rheumatoid arthritis with ACTH or cortisone have failed to change the beneficial effects. However, it is of interest that preliminary experiments in the rat, an animal which is resistant to the development of vitamin C deficiency, show that wound healing is not modified by the administration of cortisone in a dose of 1 mg per day per 100 g of rat. It is noteworthy also that the rat is remarkably resistant to histamine while the rabbit is a histamine-sensitive animal. Rats

in general are refractory to the pharmacological action of most drugs and it is possible that the dose of cortisone was too low to affect wound healing. Further studies are in progress to clarify this species difference as well as to attempt to "titrate" the minimal dosage of cortisone necessary to inhibit the growth of granulation tissue in the rabbit.

*Summary.* 11 dehydro 17 hydroxycortisone (Compound E of Kendall, cortisone) administered to rabbits in large doses caused a delay in the development of all elements of connective tissue. It is suggested that the action of cortisone and ACTH on the "mesenchymal diseases" is due, at least in part, to "inhibition of reactivity" of the connective tissue. It is furthermore suggested that the inhibition of the growth of granulation tissue in the rabbit may prove of value in assay of compounds active in the treatment of "mesenchymal diseases."

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FIG. 1.

Cross-section of rabbit's ear wound, 110  $\times$ , eighth post-operative day, control rabbit.

At the base is the cartilage of the rabbit's ear. Note the great thickness of the granulation tissue, the presence of newly-formed blood vessels and the elaboration of reticulin fibrils.

in glycogen deposition in the liver. Serum protein partitions in the animals receiving cortisone were comparable to control animals. (Table I). There was no evidence of wound infection in either the cortisone-treated or control rabbits.

Histologically, the cortisone-treated rabbits at both 5 and 8 days post-operatively showed striking depression of new growth of all elements of the connective tissue. (Fig. 1 and 2). The height of the granulation tissue formed was markedly decreased. Few, if any, new blood vessels could be seen. On the eighth post-operative day, fibroblasts in compact arrangement were present in nests about old blood vessels. In the control animals the new fibroblasts were present throughout the wound and appeared to be much less compact.

Fibrils appeared between the fibroblasts and there was more ground substance as determined roughly by metachromasia with toluidine blue stains and by the Hale(7) stain.

This suppression of all new elements of the connective tissue is similar to that seen in scurvy. Plasma ascorbic acid levels in the cortisone-treated rabbits tended to be somewhat lower than the control rabbits but the levels did not reach zero in the plasma. In this connection it is of interest that low ascorbic acid levels have been observed in our clinic in three patients with Cushing's syndrome. In the production of scurvy by deficiency of vitamin C, complete disappearance of this vitamin from the plasma is found

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## ADRENALS INTACT

## ADRENALS OUT

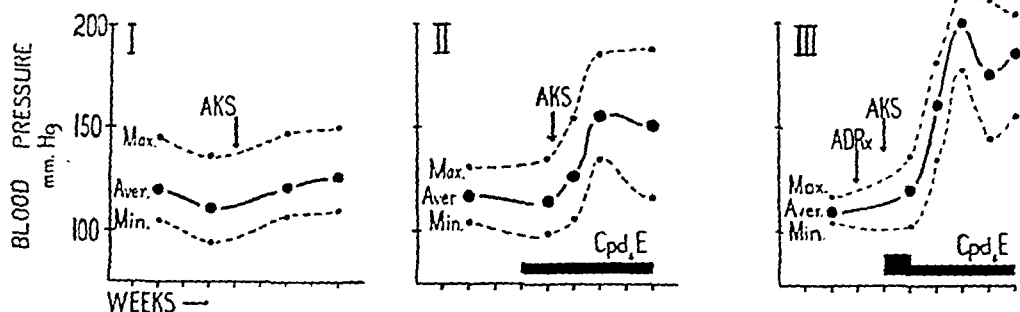


FIG. 2.

Effect of cortisone on the blood pressure of intact and adrenalectomized rats given anti-kidney serum.

Aver. = Avg weekly blood pressure of all rats in group.

Max. and Min. = Highest and lowest individual blood pressure readings each week.

AKS = Rabbit anti-rat kidney serum given at time indicated by arrow.

ADR<sub>x</sub> = Bilateral adrenalectomy performed at time indicated by arrow.

Cpd E = Cortisone. Duration of administration indicated by solid black bar.

impressive weight gain was evident within the first week after serum administration while the rats were receiving 5 mg of cortisone daily. This correlated with the development of edema in these animals. Subsequently and concomitantly with a decrease in cortisone dosage to 2.5 mg daily, the edema disappeared with a corresponding loss of weight. The differences in growth of Groups I and II are of doubtful significance, owing to the fact that the rats of Group II had attained greater growth than those of Group I when the experiment was begun.

In Fig. II the blood pressure readings are presented in graphic form. No significant change in blood pressure occurred during the 4 weeks of observation after the administration of anti-kidney serum in the group which did not receive cortisone (Group I). In the second group, which received cortisone, a moderate rise in blood pressure occurred during a similar period of time. These changes were in the same direction but less striking than had been observed previously in nephritic rats given DCA in comparable dosage.(5) In the group of adrenalectomized rats given cortisone (Group III), hypertension appeared and was of a much more impressive degree than in Group II.

In Table I the results of the chemical determinations are tabulated. Comparison of the

values obtained in the first and second groups shows no significant differences with the possible exception of the serum cholesterol, which was somewhat more elevated in the group receiving cortisone. In the adrenalectomized group (III) given cortisone, the highest value for serum sodium was obtained.

The averages and range of weights for the various organs are presented in Table II. Because of the varying size of the animals and because the groups were comprised of varying numbers of males and females, interpretation of the results is difficult. However, cardiac and renal hypertrophy was not observed in the cortisone treated groups (II and III) in the degree previously noted with DCA.(5) On the other hand, the well known atrophy of thymus and of adrenals was apparent in the cortisone injected animals.

In Table II the data pertaining to histological examination of the kidneys is included. The majority of animals in all 3 groups showed evidence of nephritis, involving glomerular, tubular and interstitial elements. Thus, cortisone failed to prevent the establishment of nephritis caused by anti-kidney serum. A comparison of the severity of the nephritis in the different groups is hazardous because of the small number of animals employed and because this form of renal damage exhibits considerable variation from animal to animal.

## The Development of Hypertension and Nephritis in Normal and Adrenalectomized Rats Treated With Cortisone.\* (17556)

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The following studies were undertaken to determine the influence of cortisone upon the development of hypertension and nephritis in rats. Observations have been made upon adrenalectomized as well as intact animals.

**Procedure.** Twenty-three 2 to 3 months old male and female rats of the Long-Evans strain were divided into 3 groups. In the 8 animals in each of the first 2 groups the adrenals were intact, while the seven animals of the third group were bilaterally adrenalectomized at the beginning of the experimental period. The animals were fed a modification of the McCollum stock diet,(1) adjusted to contain 1.7% sodium chloride. In addition, all 3 groups received 0.85% saline as drinking water. All animals were injected intravenously on 3 successive days with rabbit anti-rat kidney serum,(2) totalling 0.8 cc. Group I received only the cytotoxic serum; Group II received, in addition, daily subcutaneous injections of 2.5 mg of cortisone starting one week prior to the administration of anti-kidney serum. In Group III, cortisone was begun 8 days after adrenalectomy and immediately following the anti-kidney serum injections, in the dosage of 5 mg daily; after the first week, the daily dose was reduced to 2.5 mg. Blood pressure determinations were made at approximately weekly intervals on all rats. The indirect plethysmographic method originally described by Williams, Harrison and Grollman(3) and later modified by Sobin(4)

was employed and the results are recorded as previously outlined by these authors.(5) The growth of the animals was recorded. At the conclusion of the period of observation, 4 to 5 weeks after the administration of anti-kidney serum, the rats were anesthetized with ether, without prior fasting, then bled and sacrificed. Determinations of serum sodium were made using a flame photometer with an internal standard having an accuracy of  $\pm 1\%$ .(6) In addition, determinations of urea nitrogen were made in all groups, and, in the 2 groups of intact rats, of serum carbon dioxide content, chlorides, sugar and total cholesterol using standard methods. The weights of heart, kidneys, thymus and the adrenal glands were recorded. No adrenal tissue was found in the operated animals (Group III). Sections of kidney were fixed in Zenker's fluid and stained with hematoxylin and eosin for histological study. Sections of the pituitary were stained by Masson's technic.

**Results.** In Fig. 1 growth curves are presented. In the adrenalectomized group a most

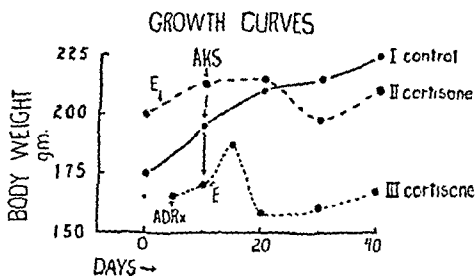


FIG. 1.

AKS = Rabbit anti-rat kidney serum.

ADR<sub>x</sub> = Bilateral adrenalectomy.

E = Date on which cortisone injections were commenced.

\* This investigation was supported, in part, by a research grant from the National Heart Institute, U. S. P. H. S., and was aided through the generosity of the Albert and Mary Lasker Foundation.

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this steroid might interfere with the development of a cytotoxic serum nephritis, the mechanism for which is likewise presumed to depend upon some form of sensitization. The results indicate that the administration of cortisone, even when given prior to the injection of antikidney serum, as in Group II, failed to prevent the development of nephritis. Since this form of nephritis represents, at least initially, the result of the introduction of preformed antibodies, these findings do not exclude the possibility that cortisone may influence the mechanisms involved in active antibody production.

In experiments with nephritic rats previously reported,(5) desoxycorticosterone acetate (DCA), when injected daily in doses of 2.5 mg over a period of weeks, produced striking hypertension. The present study indicates that cortisone, a steroid which differs from DCA in many of its physiological properties, has an effect upon the blood pressure qualitatively similar to DCA. However, in the animals with intact adrenal glands, the hypertension produced by cortisone was far less impressive than that seen previously with DCA, particularly so since in the present study a considerably higher sodium chloride intake was given. On the other hand, in the adrenalectomized rat given cortisone the hypertension is striking, and suggests the possibility that in the intact animals, the presence of other adrenal steroids tends to counteract the pressor effect of this steroid. The development of hypertension during cortisone ad-

ministration in the adrenalectomized rat with renal damage is in keeping with the previous work of Guadino.(7) Working with rats made hypertensive by a combination of unilateral nephrectomy and wrapping the remaining kidney with gauze and collodion, he observed a fall in blood pressure to follow adrenalectomy. Subsequently, cortisone injections led to a reappearance of the hypertension.

In the rats currently reported, the striking hypertension which appeared in the cortisone treated adrenalectomized group could not be correlated with a greater degree of nephritis in these animals inasmuch as renal lesions of comparable severity were observed in the other two groups.

*Summary.* 1. The administration of cortisone did not prevent the development of experimental cytotoxic serum nephritis in the rat.

2. Moderate hypertension developed in nephritic rats with intact adrenal glands when injected with cortisone, whereas striking hypertension appeared in adrenalectomized nephritic rats similarly treated.

3. The nephritic rats rendered hypertensive by the administration of cortisone presented no greater histological evidence of renal damage than did normotensive control nephritic animals.

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7. Guadino, N. M., *Rev. Argent. de Biol.* 1944, v20, 470.

## CORTISONE IN NEPHRITIC RATS

TABLE I.  
Results of Chemical Determinations.

Group	I	II	III
Adrenals	Intact	Intact	Out
Regimen	AKS	Cortisone and AKS	AKS and Cortisone
Serum sodium, mEq/L	136.4*	139.2*	143.5†
Serum CO <sub>2</sub> content, mEq/L	23.0*	24.8*	—
Serum chlorides, mEq/L	98.0*	98.2*	—
Serum sugar, mg/100 cc	174.0*	164.0*	—
Serum urea nitrogen, mg/100 cc	22.0*	24*	22†
Serum cholesterol, mg/100 cc	282*	330*	—

AKS = Rabbit anti-rat kidney serum.

\* Results based on pooled sera from all animals.

† Results based on average of individual determinations on 6 animals.

TABLE II.  
Body and Organ Weights, Incidence of Nephritis.

Group	I	II	III
Adrenals	Intact	Intact	Out
Regimen	AKS	Cortisone and AKS	AKS and Cortisone
No. of rats in group	8	8	7
No. of ♂/♀	4/4	5/3	5/2
Body wt at sacrifice (g)			
Avg	225	210	162
Range	170-346	152-265	145-179
Kidney wt (g)			
Avg	2.71	2.76	2.46
Range	1.95-3.36	1.94-3.68	1.50-4.24
No. of animals showing nephritis	7/7†	5/6†	5/7
Heart wt (g)			
Avg	.89	.91	.82
Range	.77-.98	.75-1.13	.66-1.18
Thymus wt (mg)			
Avg	141	46	49*
Range	105-173	21-87	38-61
Adrenals wt (mg)			
Avg	52.8	31.1	
Range	42.8-67.6	18-44.4	

AKS = Rabbit anti-rat kidney serum.

\* Avg of 6 animals.

† Tissues of remaining animals lost.

The lesions appeared to be of no greater severity among the two hypertensive groups (II and III) than among the normotensive group.

Dr. Philip E. Smith, who was kind enough to examine the pituitary glands of these rats for evidence of hyalinization of basophilic cells as described by Crooke in Cushing's Syndrome, reported no significant differences between con-

trol and cortisone injected animals. However, he has emphasized that the histological differentiation of rat pituitary gland is not wholly satisfactory.

*Discussion.* Cortisone has been reported to cause striking remissions in certain diseases in which antigen-antibody reactions are believed to play a role. The present study was designed to investigate whether the injection of

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